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COLD PRECIPITABLE SERUM GLOBULINS ("COLD FRACTIONS," "CRYOGLOBULINS") IN SUBACUTE BACTERIAL ENDOCARDITIS

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Jerusalem, Israel

SPONTANEOUSLY appearing precipitates are occasionally observed in sera; most of these, excluding bacterial contaminations, tend to occur on standing in the cold (4° C. to 11° C.) for various periods of time. Yet such precipitation has also been observed at 32° C. and 33° C.[1-3] As a rule, these precipitates redissolve on warming to 37° C., but apparently precipitation is irreversible in some cases.[4] The amounts encountered in various sera differ greatly, and so does the pathophysiologic importance attributed to them. These precipitates are essentially proteins; those analysed so far have been found to belong to the category of euglobulins,[5] as determined by chemical methods; to resemble gamma-globulin[4, 6, 7] when studied by electrophoresis and as to their solubility, ultraviolet absorption spectrum and nitrogen content, and to show "an amino-acid distribution suggestive of beta- or gamma-globulin."[2] Lucey and his co-workers[8] found the serum of a patient which formed a gel at 27° C. to contain a large amount of beta-globlin, which fraction was greatly reduced when this gel was removed; serologically, however, this protein cross-reacted with antiserum to normal gamma-globulin.

Abrams, Cohen, and Meyer[9] obtained a cryoglobulin from the lymph nodes and from the serum of a case of lymphosarcoma. These two samples were shown to possess identical physical properties characteristic of beta-globulin as to sedimentation constant and electrophoretic mobility.

A small number of reports have appeared describing the occurrence of cold precipitable globulins in various disorders, mainly in conditions accompanied by hyperglobulinemia. Such globulins have been discovered in some cases of multiple myeloma[7, 10] (lit. see also Lerner and Watson[11] and Barr and co-workers[7]), polyarteritis nodosa,[4, 12] in rare instances of liver diseases,[7, 13, 15] lupus erythematodes,[7] Kala-Azar,[13] rheumatoid arthritis,[14] malaria,[13] and subacute bacterial endocarditis.[11, 13]

The presence of cold precipitable globulins in large amounts seems to produce, albeit rarely, a syndrome of sensitivity to cold, manifested by purpura,

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progressive anemia, Raynaud phenomena, occasional ulcerations,[13] and thrombotic manifestations, as suggested by Barr and co-workers.[7] This syndrome, when it appears, is usually a complication of multiple myeloma.

When in 1942 Stein and Wertheimer[5] reported the occurrence of a "cold fraction" (as they termed the cold precipitable globulin) in dogs as well as in human beings infected with Kala-Azar, they also mentioned the observation of this fraction in five out of seven cases of subacute bacterial endocarditis. Twenty sera were then investigated. In a later communication[13] they reported eight more cases of subacute bacterial endocarditis in all of which a cold fraction was found. Of seventeen tests performed, sixteen had shown positive results. The authors emphasized that the cold fraction in this disease is meager in comparison with the one encountered in Kala-Azar and "only in exceptional circumstances accessible to quantitative estimation." Lerner, Barnum, and Watson[6] also found six instances of cryoglobulinemia in subacute bacterial endocarditis when performing a search for the incidence of these proteins in the sera of 121 patients with various pathological conditions. In 1947, when undertaking a survey study of the incidence of these globulins at the Montefiore Hospital, New York City, one of us (F.D.) examined, within a series of 245 sera, sera from three cases of subacute bacterial endocarditis. Two gave positive results and one, an arrested case, gave a negative result. Lepow and associates[4] also found an instance of subacute bacterial endocarditis which showed a spontaneously precipitable protein (their Case IV).

It is the purpose of this communication to report the incidence and behavior of this "cold fraction," and to correlate this finding with certain clinical, biochemical, and bacteriologic data in a series of cases of subacute bacterial endocarditis.

MATERIAL AND METHODS

Most of the cases of subacute bacterial endocarditis as observed in medical departments 'A' and 'B' of our hospital in the years 1943 to 1950 were included in this study, except for those whose records were inaccessible. The clinical diagnosis of the disease was based on the usual criteria and, especially when no confirmatory bacteriologic evidence could be obtained, the diagnosis was accepted only in the presence of the classical clinical picture of the disease, including embolic phenomena. In the course of these years we were impressed by the difficulty of obtaining a high proportion of positive blood cultures for *Streptococcus viridans* in this disease, in spite of apparently correct bacteriologic technique. On the other hand, on several occasions nonhemolytic streptococci, *Staphylococcus aureus* or *Staph. albus* appeared in the cultures of some of our cases. A case of subacute bacterial endocarditis caused by *Hemophilus paramelitensis*, grown seven times from the blood of a patient with bicuspid aortic valves, is included.

Sera to be tested for the presence of cold precipitable globulins were separated from the clot as soon as possible in order to avoid precipitation of these globulins with the clot. Since the type of cryoglobulins dealt with here precipitates only at rather low temperatures (5 to 7° C.), only in very exceptional cases small amounts of these globulins might have precipitated during the process of clot formation. For that reason, and since most of this work was performed at rather high environmental temperatures, it was not found necessary to keep the blood at 37° C. during the period of clot formation.

About 1 ml. of the serum was put into the refrigerator. The appearance of an opacity ("traces") or a precipitation at the bottom of the tube (positive "cold fraction") was looked for after twenty-four and forty-eight hours. A precipitation which would not dissolve on warming was not considered as a cold precipitable globulin.

TABLE I. RESULTS OF DETERMINATIONS OF COLD FRACTION, DILUTION FRACTION, EUGLOBULIN, AND GLOBULIN IN 50 CASES OF SUBACUTE BACTERIAL ENDOCARDITIS, WITH AND WITHOUT A COLD FRACTION DURING THEIR OBSERVED COURSE

	COLD FRACTION				DILUTION FRACTION				EUGLOBULIN			GLOBULIN		
NO. OF CASES SHOWING A COLD FRACTION	NO. OF SPECIMENS EXAMINED	POSITIVE	TRACES	NEGATIVE	NO. OF SPECIMENS EXAMINED	POSITIVE	TRACES	NEGATIVE	NO. OF SPECIMENS	INCREMENT	NORMAL	EXAMINED	INCREMENT	AVERAGE VALUE
42	161	89	22	50	123	74	41	8	128	111	17	44	34	3.28 Gm. per cent
NO. OF CASES NOT SHOWING A COLD FRACTION														
8	19			19	16	3	8	5	18	10	8	11	10	3.84 Gm. per cent
Total: 50 cases														

The "dilution fraction" test was performed in the following way: 0.2 ml. serum was diluted with 3 ml. distilled water (1:15). The resulting turbidity, if any, was observed immediately and after twenty-four hours and graded as traces or plus. Incidentally, this technique is different from the one described for the "dilution turbidity test,"[19] and we consider its results to be less valuable than the ones obtained with this latter test.

Euglobulin values were obtained by adding 3 ml. sodium sulfate 14 per cent to 0.2 ml. of serum. The resulting precipitation was observed immediately and after twenty-four hours and graded as traces or plus.

RESULTS

Altogether 50 cases of subacute bacterial endocarditis were investigated. One hundred eighty cold fraction tests were performed and in these 89 serum specimens showed cold precipitable globulins, 22 only traces, and 69 were negative. Only eight cases of the 50 did not show such a globulin at any time during their course as observed in the hospital.

Of 139 sera examined 77 specimens showed positive "dilution fractions," and 49 showed only traces. In 123 sera euglobulin was found to be increased; in 44 of the 55 sera examined (of 37 cases) total globulin exceeded 2.5 Gm. per cent (average globulin value 3.36 Gm. per cent; range betwen 1.56 Gm. per cent and 5.87 Gm. per cent). The average globulin value for the "cold fraction positive" group was 3.28 Gm. per cent, for the "cold fraction negative" group 3.84 Gm. per cent. These results are summarized in Table I.

With a view to determining whether the rather frequent appearance of the cold fraction in this disease might be a function of its lengthy course rather than a semispecific attribute of the disease, Table II was drawn up, summarizing the forty-two cases wherein a positive cold fraction has been noted at one time or another. It relates the positive and negative findings to the onset of the disease as well as to the bacteriologic findings, to the treatment administered and to the final outcome of the case. In twelve cases, at least traces of this fraction, but usually a positive finding was obtained in what appeared to be the first month of the disease; obviously the exact date of onset of subacute bacterial endocarditis cannot always be definitely ascertained. Most of the patients did not reach the hospital early enough in their course to make more results available within the first month of disease.

Although disappearance of a cold fraction could be observed in some patients who recovered from the infection, this sequence could not be demonstrated frequently enough to make it a valuable finding from a prognostic point of view. On the other hand, it may be argued that not enough tests have been performed during the period of recovery to draw any conclusions on this issue of a positive or negative nature.

No difference as to clinical, bacteriologic or biochemical data could be observed between the group of cases which did exhibit the cold precipitable globulins and the group which did not. Furthermore, no relation could be established between the appearance of a cold fraction and the bacteriologic findings: positive findings were encountered where streptococcus viridans, nonhemolytic streptococci, *Staphylococcus aureus* or *Staph. albus*, or *Hemophilus parameliten*sis

were cultured from the blood. This holds true for the "dilution fraction" and euglobulin as well which usually behaved in a manner analogous to the cold fraction.

DISCUSSION

It becomes evident that the incidence of cold precipitable globulins in subacute bacterial endocarditis is rather large (84 per cent of the cases, 61.6 per cent of determinations) in this series, one reason being that repeated examinations were carried out. Furthermore, the group of sera containing cryoglobulins contains a higher proportion of sera showing a "dilution fraction" and increased euglobulin content, but this does not hold true for the average globulin content.

Since 42 out of 50 cases of subacute bacterial endocarditis and thereof more than one-half of the sera investigated for the presence of a cold precipitable globulin showed such a protein to be present, one might ask what is the theoretical and practical importance of this finding. Globulin disturbances in this disease have been previously reported. Libman and Friedberg[16] mentioned hyperglobulinemia as a frequent finding in subacute bacterial endocarditis; this seems to be borne out also by our material. Carter and MacLagan[17] have found the thymol turbidity test to be positive in many instances of the disease; Kleeberg and Einhorn[18] have found that the Weltmann coagulation band lacks the shift to the left commonly observed in diseases of an inflammatory nature. The dilution turbidity test described by one of us,[19, 20] as well as the dilution fraction reported here, are frequently positive in the disease. The euglobulin content is apparently increased in a high proportion of cases as reported above. In our material twenty-seven specimens showed a positive formol gel reaction (out of thirty-one tests performed).

The appearance of an abnormal globulin pattern in subacute bacterial endocarditis, regardless of the particular microorganism involved, seems to represent a facet of a particular response on the part of the host, appearing at a comparatively early time in the course of this prolonged disease. It thus adds a point to the concept of those who consider this disease a special type of reaction of a predisposed host to pathogenic microorganisms of low virulence.

The presence of cold precipitable globulins has been demonstrated in various, mostly chronic, disorders. As a recent survey has shown,[14] only in a small number of diseases such as Kala-Azar, multiple myeloma and polyarteritis nodosa, but also in subacute bacterial endocarditis, as described in this paper, this finding has been so far reported in a significant proportion of cases which may lend it contributory diagnostic value. In the absence of characteristic symptoms or pathognomonic findings the diagnosis of subacute bacterial endocarditis, particularly in its early stage, may present difficulties mainly as to distinguishing it from active rheumatic heart disease. It is in these cases that the finding of cold precipitable globulins has been helpful in our past experience.

SUMMARY AND CONCLUSIONS

Cold precipitable globulins ("cold fractions" or "cryoglobulins") are fairly frequently found in subacute bacterial endocarditis: their presence could be

TABLE II. RELATION OF COLD FRACTION TESTS TO LENGTH OF DISEASE, TREATMENT, BACTERIOLOGICAL FINDINGS AND OUTCOME

PATIENT	MONTHS 1	2	3	4	5	6	7	8	9	10	11	12	CULTURES	TREATMENT	OUTCOME
D. P.		–	+		+								neg. 9×	S	Dec.
E. M.	++	+											Staph. Aur. 7×	S P 5.2*	Dec.
S. H.				+ –	–								Strep. virid. 3×	S P 5.2	Dec.
H. A.		+	– +										neg. 8×	S	Dec.
M. S.		tr	+	–	–	–							Strept. virid. 4×	S P 2.3	Rec.
S. L.				+									Staph. Aur. 2 ×	S	Dec.
M. H.			+	+									Strept. Anhem. 1×	S	Dec.
Y. B-D.			+	–					+	+tr			Staph. Aur. 7×	S P 22.5	Rec.
L. D.			trtr										neg.	S	Dec.
A. V.	++		+										neg.	S	Dec.
B. P.		+	+										neg.	S	Dec.
N. G.		–	–	++	+								Staph. Aur.	S	Unknown
S. S.				+tr									Strept. Vir.	S	Dec.
J. L.						+	++						neg.	S	Dec.
H. K.		–			+								H Paramelit. 7×	S P 7.3	Dec.
S. E.	–	tr–											Strept. vir. 3×	S P 4.8	Rec.
B. B.		+	++	+	++	+	++	+	+				Staph. Aur. 1×	S	Dec.
Y. D.		–	–	+	+–								Strept. vir. 3×	S P 15	Dec.
M. K.	–tr	–	–									–	Strept. Anhem. 2×	S P 3.1	Rec.
S. L.	– –						+			–			Strept. vir. 3×	S P 9.6	Dec.
J. A.					+								Strept. faecal. 1×	S P 11	Unknown
S. M.	tr	–			+	+							Staph. Aur. 1×	S P 8.7	Dec.
V. P.	+	++	+	++	tr	– –	–tr						neg. 14×	S P 307.5 A 17.5 Gm.	Rec.
Z. R.	+	–tr	–	–	–								Staph. Aur. 1×	S P 216	Rec.
J. B.	–	+											Strept. anhem.	S P 216	Rec.
S. E.		+tr	tr+	+									neg. 3×	S P	
S. H.	+	trtr											neg. 5×	P 14.5	Rec.
Ch. S.	++	+	+ –										Strept. vir. 1×	S P 42	Rec.
Y. S.									tr+	+ –	–		Staph. albus 7×	S P 57.8	Rec.
Y. Sh.	tr+	–tr					+			+	+		Strept. vir. 1× Strept. anhem. 1× Staph. alb. 3×	S P 18 Str. 37 Gm.	Rec.

B. P.									13	14	15			
E. F.		++	+						−	tr	+	Staph. aur. 2×	P 21	Rec.
Y. S.						+	+					Staph. aur. 1×		Rec.
B. Pa.				−	+							Staph. aur. 1×		Rec.
N. P.	++	tr−	−	−	−						•	neg. 2×	S	Dec.
A. B.	−	−	−		−							Strept. vir. 2×	P 32.6	Dec.
												Strept. anhem. 1×	P 52	Rec.
S. S.					+	−								C. H. F. Dec.
												neg. 9 ×	S P 21.3	Dec.
A. M.									14	15	20			
M. S.					+				++	tr	−	Staph. aur. 2×	P 16.5	Rec.
												neg. 3×	P 6.2	Dec. H. F.
A. S.											18			
J. H.	− +	++	++	+							+	Staph. aur. 2×	P 16	Dec. H. F.
												Strept. vir. 1×	P 112	
R. P.	++	tr	−										Aureomycin, 70 Gm.	Rec.
												Strept. vir. 7×		
												Strept. anhem. 1×	P 84	Rec.

S = Sulfonamide group; P = Penicillin; A = Aureomycin; Str. = Streptomycin; C.H.F. = congestive heart failure; Dec. = deceased; Rec. = recovered.

*5.2 = 5.2 Million Units.

demonstrated in more than half (61.6 per cent) of the 180 sera examined in 50 cases of this disease, and only eight of the 50 cases studied did not show such a globulin at any time during the observed course. This finding is frequently associated with a positive "dilution fraction" as well as an increase in total globulin and euglobulin content of the serum. Of 55 determinations of serum globulin, 44 showed an increase in globulin content over 2.5 Gm. per cent (average globulin value 3.36 Gm. per cent).

Since these globulins occur not only in cases wherein *Streptococcus viridans* has been demonstrated as the causative microorganism, their appearance may well be considered as a part of the response of the host to various microorganisms in a manner particular to this disease. These cold precipitable globulins have been found in a number of other conditions characterized by hyperglobulinemia, but in no disease state, except possibly in Kala-Azar, polyarteritis nodosa and multiple myeloma, have they been demonstrated in so high a proportion of cases.

The demonstration of their presence in the serum, a technically very simple procedure, may occasionally be helpful in the diagnosis of subacute bacterial endocarditis before unequivocal clinical and bacteriologic evidence has been obtained.

Our thanks are due to Prof. E. Wertheimer, in whose laboratory the biochemical examinations were performed, for his help and valuable advice; to Prof. J. Kleeberg for his continued interest in this study; and to Prof. M. Rachmilewitz for his permission to use material of the Medical Department "B."

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THE EFFECT OF THE "THROMBOCYTOPENIC FACTOR" OF IDIOPATHIC THROMBOCYTOPENIC PURPURA ON PLATELET LEVELS AS MEASURED BY DIRECT AND INDIRECT METHODS

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A FACTOR has been demonstrated in the plasma of six patients with idiopathic thrombocytopenic purpura which caused a prompt fall in the platelet levels as measured by the indirect method. No decrease in the circulating platelets was noted by the direct citrate method. The plasma was transfused into nonthrombocytopenic human subjects.

The current concepts of the etiology of idiopathic thrombocytopenic purpura include the following: increase in platelet lysis[1]; increase in phagocytosis of platelets[2]; suppression of megakaryocytic activity[3, 4]; and the presence of platelet agglutinins.[5] Recently Harrington and associates[6] have demonstrated the presence of a thrombocytopenic factor in the blood of eight of ten patients with idiopathic thrombocytopenic purpura. The indirect method of Dameshek[7] was used for evaluating the circulating platelets. The observations in this report confirm their studies. In addition, our results show a wide discrepancy in the platelet values of indirect and direct methods. Unfortunately, in our series no true thrombocytopenic purpura was produced such as occurred in two of the eight cases of Harrington and associates[6] so that no true purpuric effect could be studied by the methods used in our laboratory.

METHODS

The criteria of Wiseman, Doan, and Wilson[2] were used in the diagnosis of idiopathic thrombocytopenic purpura. The blood from six patients with idiopathic thrombocytopenic purpura was collected in acid citrate dextrose solution and the plasma removed. Two hundred fifty cubic centimeters of plasma were injected intravenously into nonthrombocytopenic subjects. Four recipients were normal, one had infectious hepatitis, and one was convalescing from bronchopneumonia. The platelets were counted at intervals by the indirect method of Dameshek,[7] and a modification of the Rees and Ecker direct method[8] as recommended by Tocantins,[9] using 3.6 per cent sodium citrate solution in distilled water to which 0.2 ml. of formaldehyde (neutral 40 per cent) may or may not be added to 100 ml. of solution. Our experience has shown us that the results are the same with or without the formaldehyde and we prefer not to use it. If stain is desired, 0.05 Gm. of brilliant cresyl blue is added (original Rees and Ecker method), the stain not being necessary except for the novice.[9] Normal platelet values as measured by the Dameshek indirect method range between 500,000 and 900,000 per cubic millimeter, and by the modified Rees and Ecker direct citrate method be-

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tween 225,000 and 350,000 per cubic millimeter. No control series was observed, as Harrington and associates[6] have adequately shown that no decrease in the platelets (Dameshek method) occurred in seven patients receiving blood from normal healthy donors.

RESULTS

The clinical and laboratory data of the patients with idiopathic thrombocytopenic purpura (ITP) are given in Table I.

The plasma from the six ITP subjects produced a prompt fall in the circulating platelets as measured by the indirect method, and no decrease as evaluated by the direct citrate method. The plasma was administered intravenously in one hour and an immediate platelet count was obtained. The "thrombocytopenic" effect was noted immediately in all but one recipient. In this recipient an effect was noted one and one-half hours later. The recipients were carefully evaluated for reactions following the intravenous administration of the plasma and none occurred.

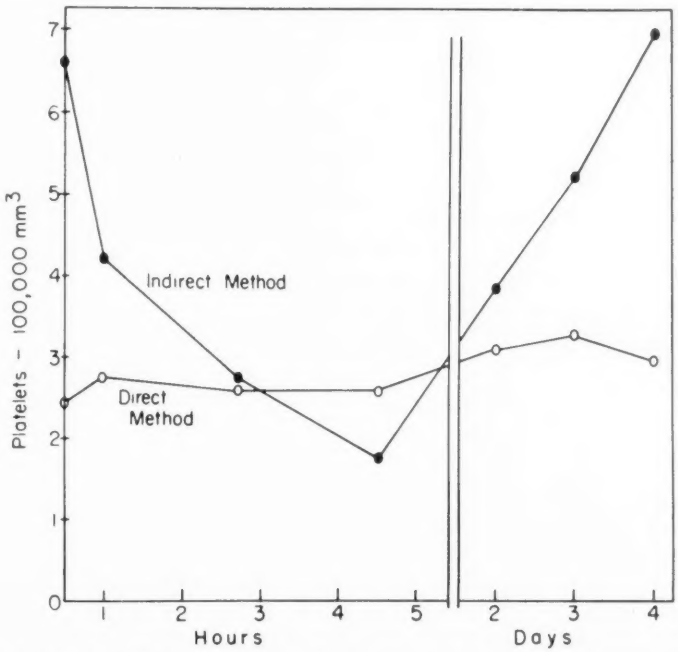

Fig. 1.—The effect on the circulating platelets of a nonthrombocytopenic subject after transfusion of 250 ml. of plasma obtained from a patient with thrombocytopenia (Case 3, Table I). A marked decrease occurred as measured by the indirect method. No significant drop occurred as evaluated by the direct citrate method.

The typical response of a normal nonthrombocytopenic subject to the plasma obtained from a patient with idiopathic thrombocytopenic purpura is shown in Fig. 1. A prompt dramatic drop occurred in the circulating platelets as measured by the indirect method. No decrease was noted as evaluated by the direct method.

The compiled data of the effect of the plasma transfusions in the six nonthrombocytopenic recipients are shown in Figs. 2 and 3. The platelet values

as obtained by the direct and indirect methods were done simultaneously in a given subject. In Fig. 2 the platelets were evaluated by the indirect method. The decrease was prompt and marked. The time necessary for the return to normal levels varied. In Fig. 3 the platelets were counted by the direct citrate method. In no instance was there a significant decrease.

DISCUSSION

A complete interpretation of the results obtained is not possible at this time. Wintrobe[10] states that incorrect high platelet counts can be caused by fragmentation without complete dissolution. Tocantins[11] reported that it

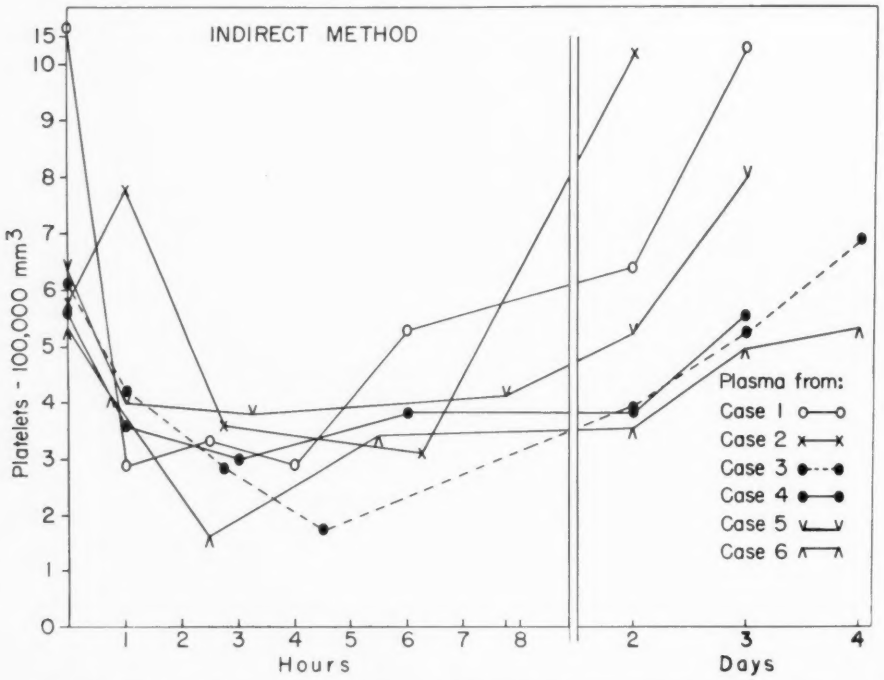

Fig. 2.—The effect on the circulating platelets of nonthrombocytopenic patients after transfusion of 250 ml. of plasma from six patients with ITP. Transfusions from 0 to 1 hour. The platelets were measured by the indirect method of Dameshek. The plasma of Case 5 was given to a postbronchopneumonia recipient and Case 6 to a patient with infectious hepatitis. All other recipients were normal.

would seem that figures above 500,000 per cubic millimeter are seldom, if ever obtained in cutaneous blood under normal conditions, and are perhaps the result of a defective technique. He further found, using an extremely careful technique, that the average number of platelets in blood from the arm was: (1) cutaneous blood—250,000 ± 7,458; (2) venous blood—310,000 ± 11,937; and (3) arterial blood—350,000 ± 13,889. The inadequacies of the indirect method have been reviewed.[9] Certainly in our laboratory the direct method has given consistent reproducible platelet values, and the indirect methods in general have not been as satisfactory.

TABLE I. DATA ON PATIENTS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA FROM WHOM PLASMA WAS OBTAINED. PLATELET LEVELS DETERMINED BY DIRECT CITRATE METHOD

PATIENT	INITIAL PLATELET COUNT (MILLIMETERS^3)	TYPE OF TREATMENT	RESPONSE OF PLATELETS (MILLIMETERS^3)
1. A. T. 22 yr. F	21,000	Cortisone failure; splenectomy	814,000
2. M. C. 17 yr. F	38,000	Splenectomy failure; ACTH response	568,000
3. B. J. 13 yr. F	10,000	Cortisone	424,000
4. F. W. 8 yr. F	14,000	Splenectomy	290,000 (7th day)
5. P. E. 9 yr. F	16,000	Cortisone	276,000 (36th day)
6. I. P. 32 yr. F	16,000	Splenectomy	250,000 (7th day)

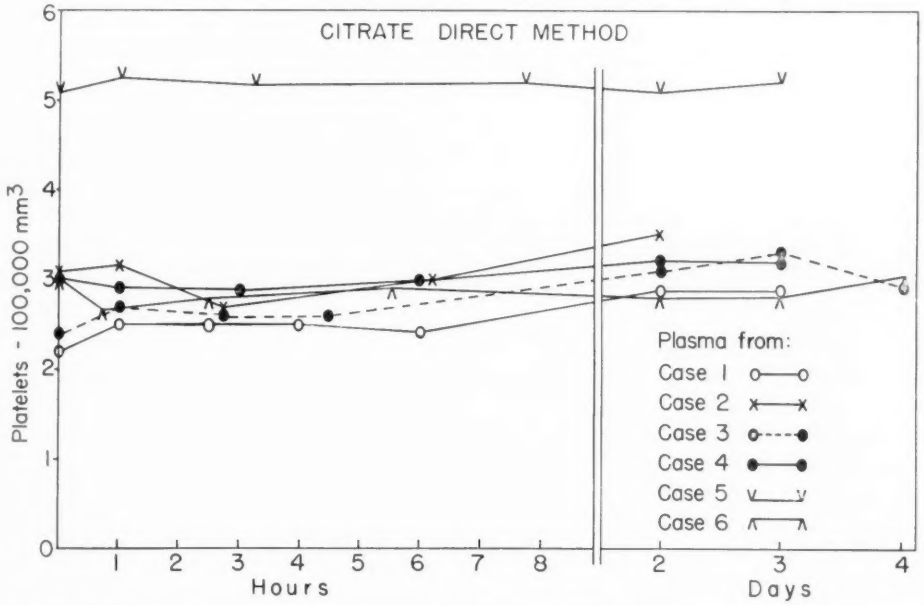

Fig. 3.—The effect on the circulating platelets of nonthrombocytopenic subjects after transfusion of 250 ml. of plasma from six patients with ITP. Transfusions from 0 to 1 hour. The platelets were measured by the direct citrate method (modified Rees and Ecker). The patients are the same as in Fig. 2, and platelets obtained at the same time as those for determination by the indirect method.

The results of the present study would seem to indicate that the indirect method is of extreme importance in evaluating a factor in the plasma of patients with idiopathic thrombocytopenic purpura. It is possible that the factor is a "platelet-fragmentation" inhibiting substance, and can be measured only by a correlation of both the indirect and direct methods for enumerating circulating platelets. The two cases of Harrington and associates[6] cannot be ignored. In these the signs and symptoms of thrombocytopenic purpura were produced by the injected plasma. Is it possible that the blood of patients with idiopathic thrombocytopenic purpura contains a factor that (1) inhibits platelet fragmentation, and (2) in greater concentrations produces a true

thrombocytopenia? This question can be answered only by an explanation of the exact cause of the differences in platelet levels as determined by the direct and indirect methods. The basic difference in the two methods is that in the direct method there is a tremendous dilution of the blood (1:200) and in the indirect method the dilution is minimal, probably about one part of whole blood and five parts counting fluid. The theoretical factor normally promoting platelet fragmentation is probably diluted by the direct method to the extent that most of its action is inhibited.

CONCLUSIONS

A factor was demonstrated in the plasma of six patients with idiopathic thrombocytopenic purpura that produced a thrombocytopenia as measured by an indirect platelet method. However, the circulating platelets as evaluated by the direct citrate method were not decreased in number. A correlation of both the indirect and direct methods of measuring the circulating blood platelets is of the utmost importance in an evaluation of the "thrombocytopenic factor" of idiopathic thrombocytopenic purpura. The factor probably inhibits platelet fragmentation.

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BONE MARROW IN IDIOPATHIC THROMBOCYTOPENIC PURPURA

Analysis of 100 Cases with Reference to the Prognostic Significance of Eosinophils and Megakaryocytes

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INTRODUCTION

During the past fifteen years, since bone marrow aspirates have become more widely utilized in diagnosis, several reviews of the characteristic cellular findings in idiopathic thrombocytopenic purpura have appeared.

There are wide differences in the cellular composition from one patient to another. The most consistently observed finding has been an increased number of megakaryocytes with a tendency toward immaturity and a failure of platelet formation. These changes in the megakaryocytes have been used as confirmatory evidence for idiopathic thrombocytopenic purpura in conjunction with other typical clinical and hematologic manifestations.

The course of the disease can vary from slight to no clinical findings and thrombocytopenia of only a few weeks duration to a severe form which may lead to rapid death from internal hemorrhage. Splenectomy, the most generally useful method of treatment to date, has an unpredictable effect in individual cases. Most patients experience immediate relief, while others may not be benefited at all. This being the case, a reliable index of prognosis would be helpful to the practitioner.

In looking for such a guide, Schwartz[15, 16] has reported a tendency toward greater bone marrow eosinophilia in cases with a favorable outcome than in those with an unfavorable outcome following either conservative management or splenectomy. In this regard, he postulates an allergic etiology in the cases with favorable outcome to account for this observation.

Others have implied that the presence of many immature megakaryocytes is of good prognostic significance in this disorder.[2, 9, 11, 12]

To evaluate critically the importance of marrow eosinophilia and megakaryocytes in the diagnosis and prognosis of this disease, and to assess the clinical value of their quantitation in individual cases, bone marrow smears from 100 cases of idiopathic thrombocytopenic purpura have been analyzed and compared with a similar series of normal subjects. The criteria for diagnosis in these cases included thrombocytopenia (less than 100,000 per cubic millimeter and the absence of diseases such as leukemia or lupus erythematosus on bone marrow examination and during a follow-up period of at least one year.

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SUBJECTS AND METHODS

Patients with idiopathic thrombocytopenic purpura have, for the most part, been observed in the hematology clinic at the University of Illinois over the past ten years. There were 34 males and 66 females, ranging in age from 13 months to 73 years. The distribution by ages was essentially similar to that in Schwartz's series, and is presented in relation to outcome in Table I. Thirty-six of the cases were first seen while in the acute phase of the disease, while 64 were chronic. Forty-six of the 100 patients had splenectomy,* while the remainder were treated conservatively.

Subjects of the control group ranged in age from 11½ months to 69 years, and were about equally divided as to sex. They had no apparent disease at the time of marrow examination, and were not known to suffer from clinical allergies.

TABLE I. FINAL OUTCOME IN RELATION TO AGE

AGE (YEARS)	NO. OF CASES	NO SPLENECTOMY			SPLENECTOMY		
		SPONTANEOUS REMISSION	CHRONIC PURPURA	DIED	SUCCESSFUL	UNIMPROVED	DIED
0-15	26	19	1	–	6	–	–
15-30	40	8	5	1	24	1	1
30-45	20	5	4	–	7	3	1
45+	14	3	7	1	2	–	1
All cases	100	35	17	2	39	4	3

Sternal marrow was aspirated, concentrated, and smeared according to the technique previously outlined by Limarzi.[7, 8] For marrow study, smear technique is considered preferable to chamber counts.[1]

The relative number of eosinophils was computed from a count of 1,000 late-stage granulocytes (neutrophils and eosinophils) in the metamyelocyte and segmented stages only. Eosinophils are expressed as percentage of late-stage granulocytes. Schwartz used an index equivalent to the number of eosinophils seen in counting 1,000 such neutrophils. Results from the two different methods of enumeration are mathematically interchangeable, an eosinophil index of 50 being equivalent to a late-stage granulocyte percentage of 4.8. The prevalence of immature eosinophils was also quantitated as the percentage of early-stage granulocytes (1,000 cells) in the progranulocyte through myelocyte stages.

No satisfactory method has been advanced for the accurate counting of bone marrow megakaryocytes.[5, 14] Due to the variable tenacity with which different cell types are held in place by the reticular network, different techniques of aspiration and smear preparation give different relative numbers of these cells in normal and abnormal conditions. If a given technique is accurately repeated for each aspirate, however, valid comparisons can be made between large series of cases despite individual inaccuracies. Through study of several thousand marrows uniformly prepared, the senior author (L. R. L.) has found that the simplest and most reliable method of evaluation consists of a rapid scrutiny of each smear under low magnification (×40) and a semiquantitative estimate of the numbers of megakaryocytes as *none seen, decreased, adequate,* or *increased.* This simple technique has been used in the present report.

Differential megakaryocyte counts were based on the classification of 100 consecutive megakaryocytes from each smear into *immature, mature,* or *degenerated forms.*[10, 11] This differentiation requires scrutiny of each such cell under oil immersion in a sufficiently thin part of the smear that osmotic distortion does not alter morphology and staining qualities. In evaluation of the age of megakaryocytes, the amount, character, and arrangement of azurophil granules is of more importance than the amount of nuclear lobulation (Figs. 1 and 2).

*Weights of the spleens were recorded in 42 of these cases. In the 7 children (up to 16 years of age), spleen weights ranged from 90 to 290 Gm. Spleens in 11 of the 28 adult patients with favorable outcome and 5 of the 7 with unfavorable outcome weighed greater than 210 Gm., the upper limits of normal.[6]

Immature megakaryocytes include the blast forms and promegakaryocytes. Megakaryoblasts have a more basophilic cytoplasm and increased density of the chromatin strands as compared to their precursors the myeloblasts (hemocytoblasts). There are no granules demonstrable at this stage. Promegakaryocytes, however, show increasing azurophil granulation of the cytoplasm and maturing of the nucleus, but no platelet formation.

In mature megakaryocytes, the granules tend to form many clumps throughout the cytoplasm. Actual platelet formation may be evident. In most cells at this stage, the

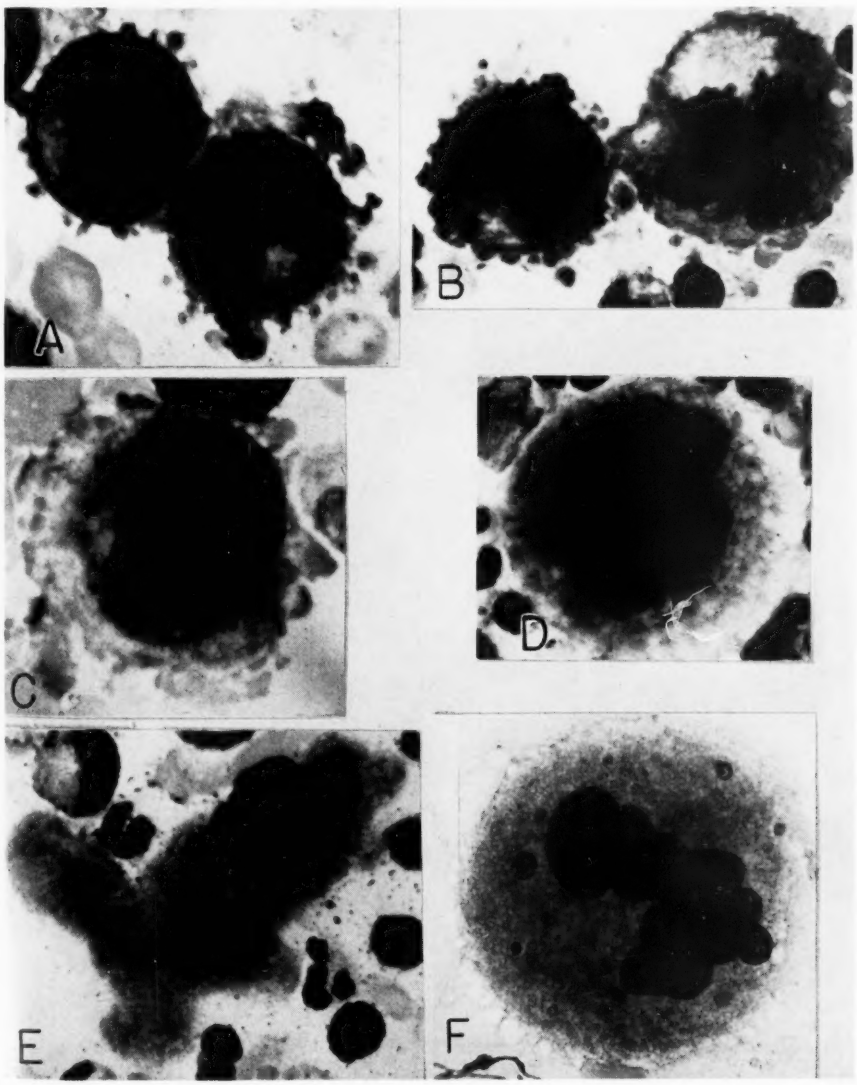

Fig. 1.—*A* to *C*, *Immature megakaryocytes*. *A*, Megakaryoblasts with relatively scant basophilic cytoplasm, no azurophil granulation, and slight coarsening of chromatin strands. Even at this early stage, the nucleus may be noncircular in outline. *B*, Megakaryoblast on the left. Promegakaryocyte on the right with beginning, nonclumped azurophil granulation and increased coarsening of the chromatin net. *C*, Promegakaryocyte. *D* to *F*, *Mature megakaryocytes*. *D*, Clumping of granules and further coarsening of nuclear chromatin. *E*, Increasing amount of and irregular outline to cytoplasm. *F*, Multilobulation of nucleus.

nucleus is complexly lobulated with dense aggregates of chromatin, and the cytoplasmic outline is irregular or even ragged.

Degenerated forms are the isolated, dark-staining, pyknotic, megakaryocyte nuclei, and cells with an agranular, hyalin-appearing cytoplasm with mature nuclei.

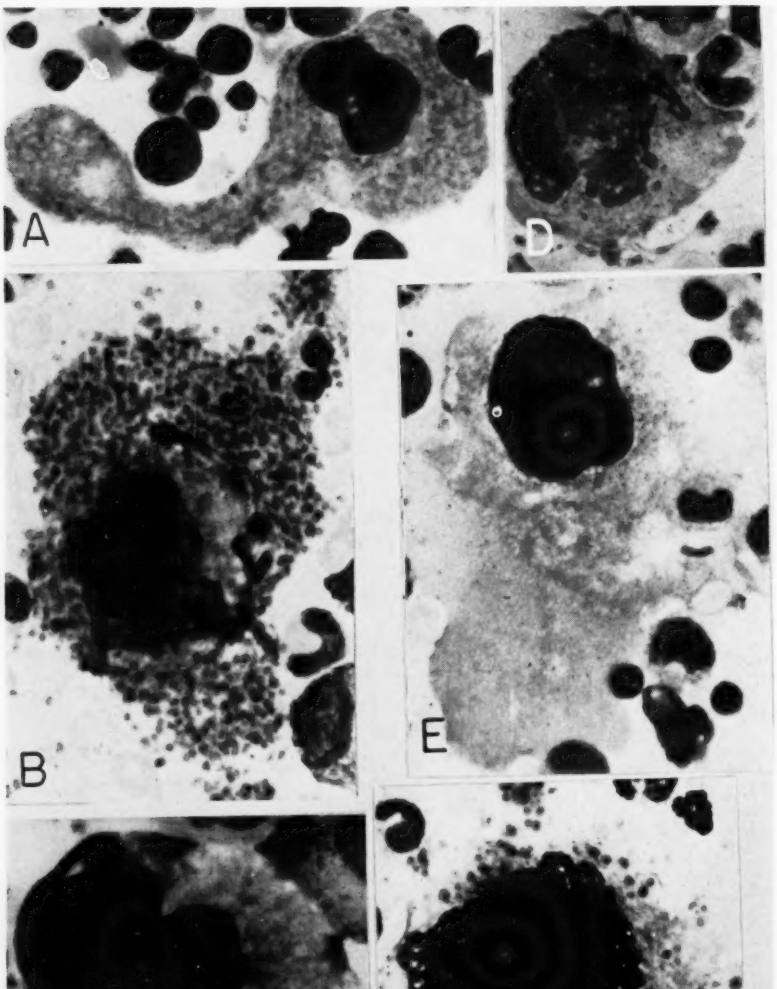

Fig. 2.—*A to B, Mature megakaryocytes. A,* Pseudopod formation. *B,* Megakaryocyte surrounded by platelets (idiopathic thrombocytopenic purpura, post-splenectomy). *C* to *F, Degenerated megakaryocytes. C,* Densely staining chromatin with loss of finer nuclear details. Agranular, hyalin-appearing cytoplasm. *D,* Degenerative fenestration of nuclear pattern with agranular cytoplasm. *E,* Pyknotic nucleus and large amount of hyalin-like cytoplasm. *F, Intermediate Form.* Nucleus is degenerating, but platelet formation is evident in the cytoplasm. This cell would be considered a mature form.

RESULTS

Outcome of the 100 cases of idiopathic thrombocytopenic purpura has been classified according to whether cases showed a so-called "high" or "low" percentage of late-stage granulocytes (Table II). Outcome was based on observation for at least one year or until death. Most cases have been followed for a

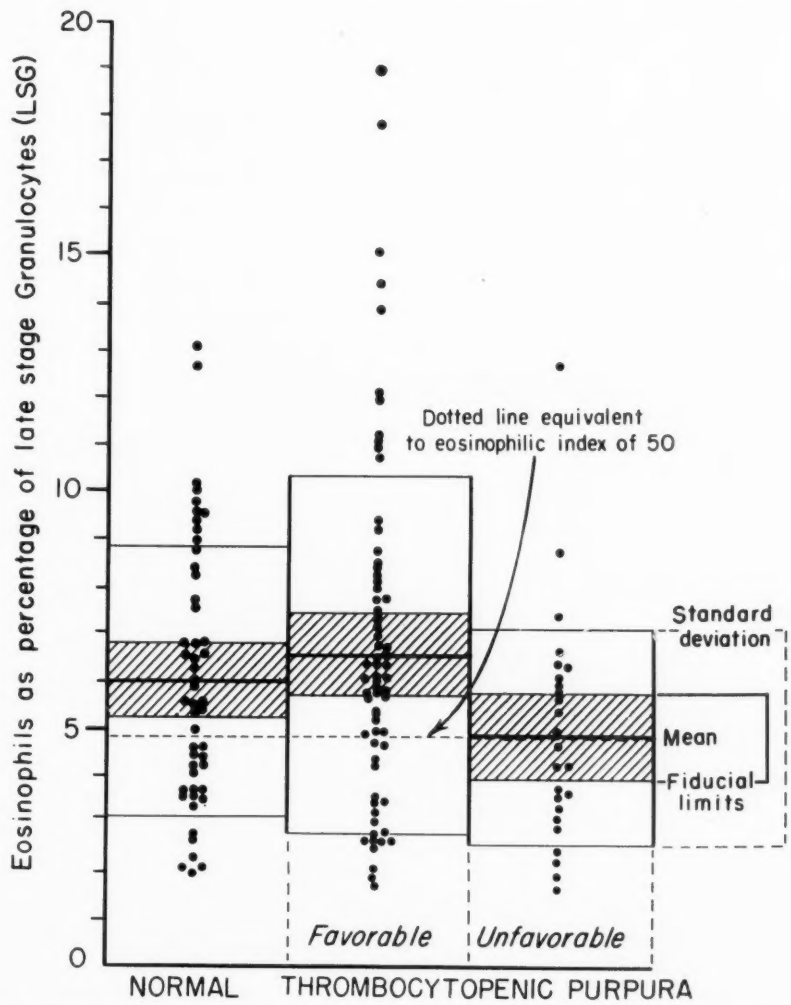

Fig. 3.—Comparison of marrow eosinophil percentages in normal subjects and in patients with idiopathic thrombocytopenic purpura having favorable and unfavorable outcomes. ("Eosinophilic index" of 50 chosen by Schwartz to divide "high" from "low" degrees of marrow eosinophilia.[15])

much longer time than one year. The figures from the present study are very similar to those of Schwartz, with the exception of cases subjected to splenectomy. In his cases with low eosinophil values, 8 of 18 patients having splenectomy died, while another 2 were unimproved. Only 1 of 21 low eosinophil

TABLE II. FINAL OUTCOME IN RELATION TO "HIGH" OR "LOW" EOSINOPHIL PERCENTAGE

			NO SPLENECTOMY			SPLENECTOMY		
		NUMBER OF CASES	SPONTANEOUS REMISSION	CHRONIC PURPURA	DIED	SUCCESSFUL	UNIMPROVED	DIED
Eosinophil per cent	*High* Over 4.8 per cent (LSG) (equivalent to Eosinophil index over 50)	61	28 (78)	8 (12)	–	21 (84)	2 (8)	2 (8)
	Low Under 4.8 per cent (LSG)	39	7 (39)	9 (50)	2 (11)	18 (86)	2 (9)	1 (5)
	Total	100	35	17	2	39	4	3

*Figures in parentheses indicate percentage of cases in the particular therapeutic and eosinophil percentage group according to outcome.

patients died following splenectomy in our series, while another 2 were unimproved. These differences could well be due to chance alone, particularly inasmuch as his differentiating index of 50 was arbitrarily selected on the basis of observed results.[15] Slight changes in this point of differentiation might make considerable difference in the distribution of the data.

A better method of comparison is obtained by a plot of the actually observed marrow eosinophil values according to outcome, and comparison of these values with those obtained from nonallergic normal subjects as is shown in Fig. 3. Outcome was pooled into two groups: *favorable* (74 cases), including those with spontaneous remission and successful splenectomy, and the *unfavorable* (26 cases), including all who died, those who continued a chronic course, and those with no improvement following splenectomy. Counts from 50 nonallergic normal subjects were used for comparison. It is seen that the scatter of results in the normal subjects and two groups of thrombocytopenic purpura patients show a large overlap. Statistical comparisons can be made through analysis of the mean, standard error of the mean, and fiducial limits* of the mean, and standard deviation according to standard techniques.

TABLE III. COMPARISON OF EOSINOPHIL PERCENTAGES IN EARLY-STAGE AND LATE-STAGE GRANULOCYTE SERIES

	LATE-STAGE GRANULOCYTES			EARLY-STAGE GRANULOCYTES		
		THROMBOCYTOPENIC PURPURA			THROMBOCYTOPENIC PURPURA	
	NORMAL	FAVORABLE OUTCOME	UNFAVORABLE OUTCOME	NORMAL	FAVORABLE OUTCOME	UNFAVORABLE OUTCOME
Number of cases	50	74	26	50	74	26
Mean	60.80	66.20	49.20	96.80	113.52	97.69
Standard deviation	28.56	37.60	22.79	41.27	68.79	48.11
Fiducial limits	52.88	57.57	39.99	85.36	97.85	78.26
	68.72	74.83	58.41	108.24	129.19	117.12

*Fiducial limits of the mean are here considered as the range within which there is 95 per cent confidence that the mean of an infinitely larger population of the same composition would fall.

The fiducial limits for the three groups have a definite overlap, indicating that no significant difference between these groups has been demonstrated.

It is noted that in the unfavorable outcome group as many observations are seen above as below the dotted line equivalent to Schwartz's eosinophil index of 50. Eosinophil percentages are generally above this line in the other two groups, although many of the values are below it. Except for a few patients with very high eosinophil values, there appears to be no difference between the cases of purpura and normal subjects.

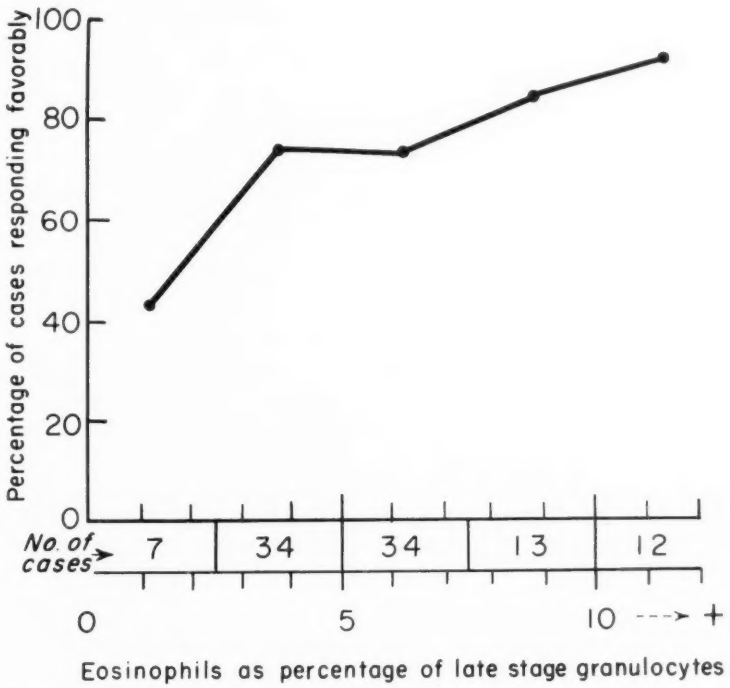

Fig. 4.—Percentage of cases having a favorable outcome as related to degree of marrow eosinophilia.

Although the mean difference between those cases with favorable versus unfavorable outcomes cannot be considered statistically significant, the evidence appears suggestive that in a much larger series a significant difference might be evident. These data may also be considered primarily according to the scale of marrow eosinophilia with percentage of favorable outcome for each level indicated as in Fig. 4. Favorable outcomes are seen in a majority of cases regardless of eosinophil numbers in the marrows, but at very high levels the chances for a favorable outcome are the greatest.

In our series, eosinophil counts were slightly higher in younger age groups in purpura patients, but this tendency was not statistically significant (Fig. 5).

Similar studies were done on the same subjects counting early-stage granulocytes. Essentially the same results were noted as with late-stage granulocytes. Comparative values are presented in Table III.

Peripheral eosinophil percentages from differential counts were compared with the prevalence of marrow eosinophils. No correlation was apparent.

Relative Number of Megakaryocytes.—Of the thirty-six cases of acute purpura, eleven had adequate numbers of megakaryocytes, 24 showed an in-

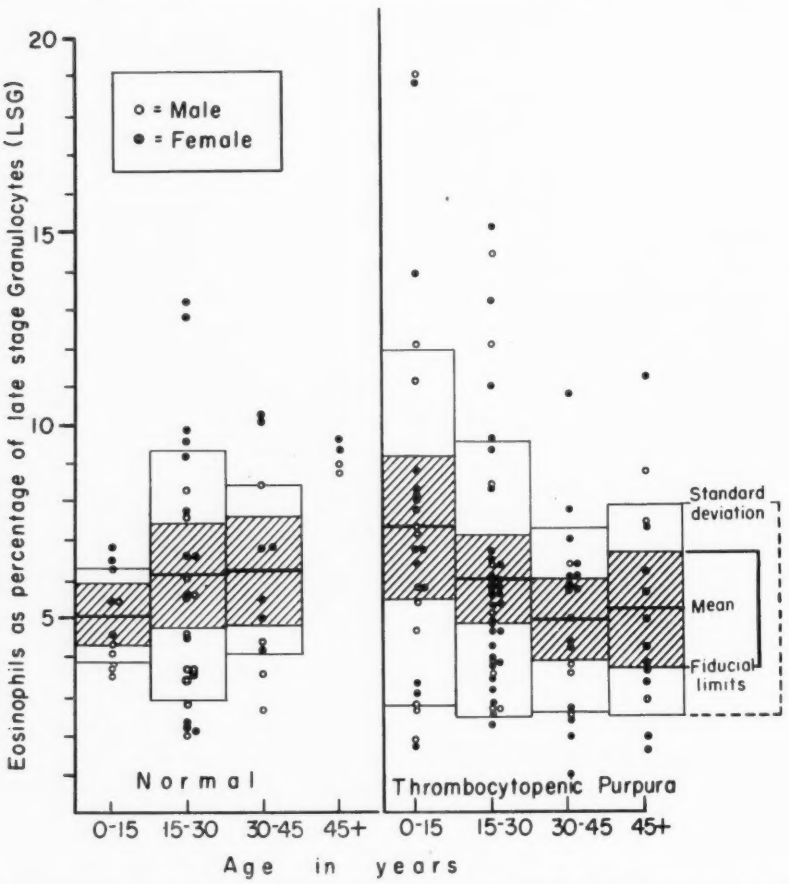

Fig. 5.—Comparison of marrow eosinophil percentages in normal subjects and in all patients with idiopathic thrombocytopenic purpura according to age.

crease, and one had none demonstrable. The absence of megakaryocytes in the aspirated marrow smear of one patient was apparently related to chance and to the technique of aspiration (this particular aspiration was not performed by the authors). The marrow was hypercellular with numerous myeloid and erythroid elements present. The patient died about one week later from a cerebral hemorrhage. On autopsy, an increased number of marrow megakaryocytes were evident, and no evidence for diseases other than idiopathic thrombocytopenic purpura with its sequelae were found.

Twenty of the 64 chronic purpura cases had adequate megakaryocytes, 40 showed an increase, and 4 had a decrease. Table IV compares the relative number of megakaryocytes to the final outcome. It is seen that a slightly greater percentage of patients with favorable outcome had increased numbers of megakaryocytes than those with unfavorable outcome. That decreased numbers of megakaryocytes are not necessarily of bad prognostic importance is attested by the fact that three such patients experienced relief following splenectomy, and one had a spontaneous remission.

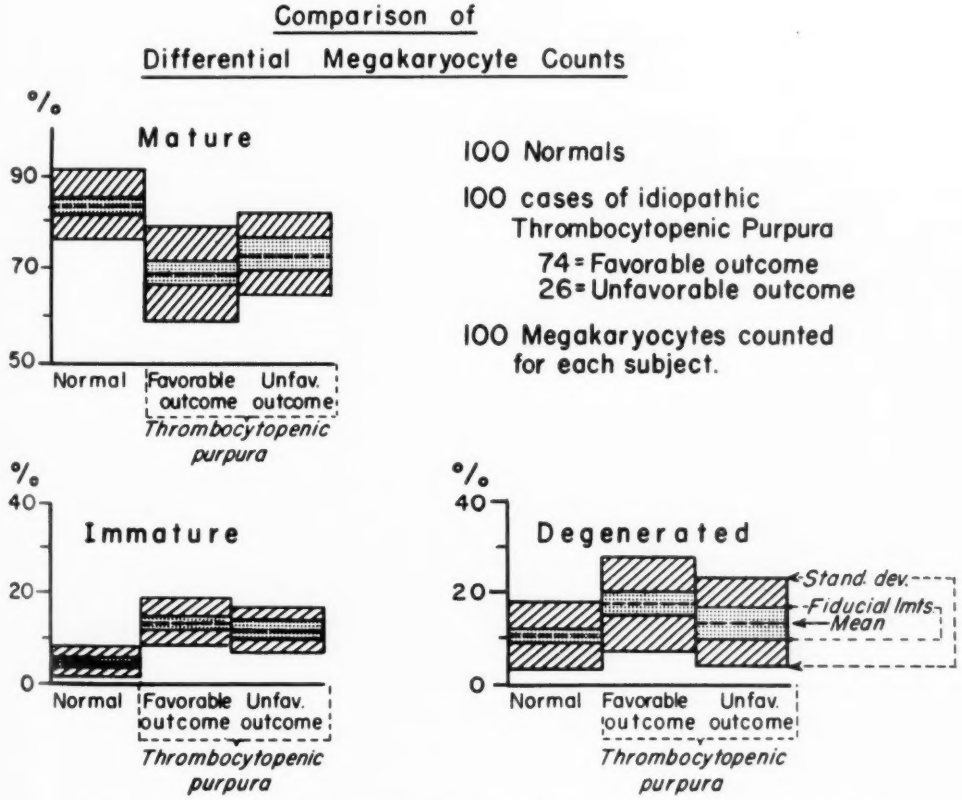

Fig. 6.—Comparison of differential megakaryocyte counts in normal subjects and in patients with idiopathic thrombocytopenic purpura having favorable and unfavorable outcomes.

Differential Count of Megakaryocytes.—Statistical analyses of the differential counts of 100 normal subjects and the 100 cases of purpura are graphically presented in Fig. 6. Significant differences between the mean percentage of mature and immature cells of the normal subjects as compared with the patients with thrombocytopenic purpura are evidenced by the definite separation between their respective fiducial limits. There is some overlap of observations, as can be seen in the one standard deviation limits, but it is not as great relatively as is that with regard to eosinophils. The slight

TABLE IV. RELATIVE NUMBER OF MEGAKARYOCYTES

MEGAKARYOCYTES	FAVORABLE OUTCOME	UNFAVORABLE OUTCOME
	Acute Purpura (36 cases)	
Adequate	7	4
Increased	18	6
Decreased	–	–
None seen	–	1
	Chronic Purpura (64 cases)	
Adequate	15	5
Increased	30	10
Decreased	4	–

differences between cases having favorable as against unfavorable outcomes are not significant. Patients having favorable outcome showed a significantly greater mean percentage of degenerated forms over the normal subjects.

DISCUSSION

Diagnostic Value of Marrow Examination in Idiopathic Thrombocytopenic Purpura.—The present study again confirms the characteristic bone marrow findings of idiopathic thrombocytopenic purpura. Approximately two-thirds of the cases had increased numbers of megakaryocytes. There was a significant increase in immature forms, 13 per cent of megakaryocytes being immature on the average, as apposed to 5 per cent for normal subjects. Likewise, the average percentage of degenerated forms was 15 per cent, as apposed to 11 per cent in the normal subjects. The percentage of mature forms was correspondingly decreased. However, many individual exceptions to these general tendencies diminish their value as reliable indices of diagnosis. No significant difference was noted between the prevalence of eosinophils in these subjects as apposed to the normals.

Certainly, no one could wish to make a diagnosis of thrombocytopenic purpura on the basis of marrow examination alone. Careful clinical and peripheral blood studies should always precede a marrow examination. Bone marrow study is nonetheless of great value in cases of thrombocytopenia: first, to rule out other diseases leading to secondary platelet depression such as leukemia or aplastic anemia; second, to add confirmatory evidence for a diagnosis of idiopathic thrombocytopenic purpura in those cases which definitely demonstrate increased numbers of immature megakaryocytes.

Prognostic Value of Marrow Examination in Idiopathic Thrombocytopenic Purpura.—The prevalence and relative maturity of megakaryocytes had no significant relationship to ultimate outcome in these cases. Several cases with decreased numbers of megakaryocytes had a favorable outcome.

With regard to eosinophils, the trend toward more favorable outcome with greater degrees of marrow eosinophilia as observed by Schwartz was confirmed. However, the arbitrary dichotomy of "high" versus "low" eosinophil percentage based on an eosinophilic index of 50 or corresponding percentage of late-stage granulocytes amounting to 4.8 is seen to be misleading. Such an index is of no practical value for separating cases destined to have good versus

those destined to have poor prognoses. Other investigators have also failed to find significant prognostic value in this test.[18] Diggs and Hewlett[3] could find no relation between eosinophilia and prognosis. In a series of 7 cases of idiopathic thrombocytopenic purpura studied by Elliott and Turner,[4] only one showed marrow eosinophilia. This patient had a chronic and severe purpura unrelieved by splenectomy and marked by cerebral hemorrhage. Newton and Zuelzer[13] reported that they found no prognostic correlations from marrow megakaryocytes or degree of eosinophilia in 47 children with idiopathic thrombocytopenic purpura. Likewise, the thesis that any considerable number of those with "high" eosinophil percentages can be considered as "allergic" thrombocytopenic purpura on this evidence alone is made untenable by the fact that normal, nonallergic subjects show essentially the same range of marrow eosinophilia. Careful scrutiny of many reported purpura cases with "high" eosinophilic indices has shown other evidence for allergic etiology only in a very few instances.[17] Through use of Fig. 4 one may get a relative estimate of prognosis in individual cases according to the percentage of marrow eosinophils. Other factors in such an estimate would include the age of the patients (very young and very old subjects generally show more favorable courses) and the manifestations of the disease up to the time of examination. Although these factors are of some value in guessing the outcome of a given case, they should never be held as contraindications to definitive therapy such as splenectomy if such a procedure appears warranted on other clinical evidence.

On Counting Marrow Eosinophils.—Schwartz states that in cells earlier than metamyelocytes, specific granules might not be unequivocally identified, and hence he chose to count only late-stage eosinophils. In our experience the uniform size, roundness, and refractile qualities of the eosinophil granules make their identity unmistakable, in spite of the fact that early stages often take on basophilic rather than acidophilic dyes. The trends of immature eosinophils as related to early-stage granulocytes were parallel to those of the mature series. In future investigation, except for ease of comparison with studies already published, there would appear to be no good reason why eosinophils at all stages of maturation should not be included in the counts. As a matter of fact, the unpredictable contribution of sinusoidal blood to the number of mature eosinophils and neutrophils would be minimized thereby.

CONCLUSIONS

Bone marrow smears from 100 cases of idiopathic thrombocytopenic purpura were compared with those of normal nonallergic subjects. The following findings were demonstrated:

1. There was no significant difference in marrow eosinophil counts between normal subjects and the cases studied.
2. Patients with this disorder on the average showed increasingly better prognoses, with progressively increasing degrees of marrow eosinophilia.

3. However, no single figure for the relative number of marrow eosinophils is of practical significance for separating cases of favorable versus unfavorable outcome.
4. There was a definite tendency toward increased number and relative immaturity of megakaryocytes in idiopathic thrombocytopenic purpura cases.
5. These megakaryocyte trends were not universally observed, and their absence or degree of occurrence appeared to have no prognostic significance.
6. Bone marrow examination is primarily of value for differentiating secondary thrombocytopenia from this disorder and as confirmatory evidence for the diagnosis of idiopathic thrombocytopenic purpura.

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VARIATIONS IN NUCLEOTIDASE ACTIVITY OF LEUKOCYTES. STUDIES WITH LEUKEMIA PATIENTS

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A NUMBER of studies have established that the leukocytes contain phosphatases, and attempts have been made to quantitate these enzymes using β-glycerophosphate or sodium phenylphosphate as substrates.[1-4] In another series of investigations, the relatively high nucleic acid content, as well as large desoxyribonucleic acid turnover values obtained from hemopoietic organs,[5, 6] have made the nucleic acid metabolism of the leukocyte a subject of considerable interest. It therefore seemed pertinent to determine the activity of nucleotidase in the various types of leukocytes. This enzyme is the phosphatase concerned in the degradation of a constituent part of the nucleic acid molecule.

It has been found in the studies to be reported here that the nucleotidase of leukocytes is an acid phosphatase with a pH optimum of 4.0 and is nonspecific in character, i.e., adenosine-5-phosphate, adenosine-2-phosphate, or adenosine-3-phosphate can serve as substrates. Most interesting has been the observation that the nucleotidase activity of the granulocyte is much greater than the lymphocyte, an implication that the metabolism of these two cell types as regards nucleic acid may be fundamentally different.

METHODS

Enzyme Sources.—Leukocytes used in these studies were obtained from the peripheral blood of patients with chronic forms of leukemia. The leukemia patients were so selected that at least 90 per cent of the total leukocyte count was characteristic of one-cell series, either granulocytic or lymphocytic. None of the patients had received therapy within a six-month period prior to their participation in this investigation.

Leukocytes were separated from the peripheral blood by the method of Gorham and co-workers[7] and centrifuged to a state of maximum packing. The cells were then homogenized in water at a dilution suitable for use in the enzyme assay.

Substrates.—Adenosine-5-phosphate (A-5-P) was obtained from Sigma Chemical Company or Schwarz Laboratories.

α and β isomers of adenosine-3-phosphate, designated as adenosine-2-phosphate (A-2-P) and adenosine-3-phosphate (A-3-P)[9] were obtained from Schwarz Laboratories.

Enzyme Assay.—In a total volume of 1.0 ml. of reaction mixture, 0.2 ml. of buffer (glycylglycine, veronal, or acetate, 0.05M), 0.1 ml. $MgCl_2$ (0.04M), 0.2 ml. of adenylic acid (0.01M in appropriate buffer) and enzyme were added, a procedure similar to that of DuBois and co-workers.[8] A 5 per cent homogenate of lymphocytes or a 2 per cent homogenate of granulocytes were assayed at 0.1, 0.2, and 0.4 ml. levels. Incubation was for 30 minutes

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at 38° C., and the reaction was stopped by the addition of 0.5 ml. 10 per cent trichloracetic acid. After centrifugation, inorganic phosphorous determinations were made on an aliquot by the method of Fiske and SubbaRow.[10] Results were recorded as μg of phosphorus liberated per milliliter of centrifuged white cells per hour. In all experiments duplicate tubes were included to which trichloracetic acid was added prior to incubation in order to obtain a substrate blank.

RESULTS

pH Optimum and Non-specificity of Leukocyte Nucleotidase.—Using A-5-P as substrate, the effect of pH on the nucleotidase activity of lymphocytes and granulocytes was determined. The results of a typical case of lymphocytic and one of granulocytic leukemia are shown in Fig. 1. The activity is recorded in terms of per cent, where 100 is the greatest amount of phosphorus liberated at any pH tested and indicates that nucleotidases of both cell types have a single optimal activity peak at pH 4.

Nucleotidases have been obtained from mammalian, reptilian, and plant sources which hydrolyze only nucleotides containing phosphate esterified at carbon 5 of ribose[11-13] and those phosphatases have been termed 5-nucleotidase. However, the available evidence as obtained in this study would indicate that the leukocyte nucleotidase is nonspecific in character, hydrolyzing A-3-P and A-2-P as well as A-5-P. In addition to identical pH optima for all these substrates, it has been found that in substrate competition experiments, when mixtures of the nucleotides were employed, the rate of liberation of phosphorus was not additive but intermediate between individual rates. It has also been found that the leukocytes will hydrolyze guanylic and cytidylic acids.

Nucleotidase Activity of Granulocytes and Lymphocytes.—Determinations of nucleotidase activity at pH 4 were carried out as outlined above in granulocytes from a series of ten patients with chronic granulocytic leukemia and in lymphocytes from eight patients with chronic lymphocytic leukemia. The results in Table I show that the nucleotidase activity as measured by rate of phosphorus liberation per milliliter of packed cells is approximately ten times greater in granulocytes than in lymphocytes. These cellular differences also pertain when nucleotidase activity is calculated on the basis of cell nitrogen.

TABLE I. NUCLEOTIDASE ACTIVITY OF LEUKOCYTES

	GRANULOCYTES				LYMPHOCYTES		
	PHOSPHORUS RELEASED/HR./ML. CELLS				PHOSPHORUS RELEASED/HR./ML. CELLS		
PATIENT	A-5-P (μG)	A-2-P (μG)	A-3-P (μG)	PATIENT	A-5-P (μG)	A-2-P (μG)	A-3-P (μG)
Tr	4,320		5,600	Du	400		480
Ho	3,840	3,680	4,960	Po	520	680	480
Do	5,670	5,670	8,160	Ko	440	360	440
Ha	4,960	5,120	8,360	Bo	640		
Ho	2,880	2,720	4,800	Ke	200		
Si	4,480	4,760	6,400	Jo	240		480
Oz	3,040		4,960	Ro	520		
Doy	2,300		4,000	Bo	210		
Ca	4,800		7,360				
Sc	3,040		4,800				

For granulocytes, an invariably higher rate of phosphorus liberation is obtained with A-3-P as substrate than with either A-5-P or A-2-P. This phenomenon might well be a reflection of the greater affinity of the enzyme for the A-3-P substrate.

DISCUSSION

Since in these studies patients with chronic types of leukemia were used, the leukocytes possessed morphologic characteristics similar to those of normal individuals, and it might be postulated that enzymic relations are also comparable. In this event, the total leukocyte nucleotidase activity in normal subjects is the resultant of a low lymphocytic nucleotidase level and a high granulocytic nucleotidase level and any direct comparison between the activity of this enzyme in normal and leukemic individuals becomes more or less meaningless. Similar circumstances would prevail with any enzyme or metabolite whose concentrations vary widely in lymphocytes as compared to granulocytes.

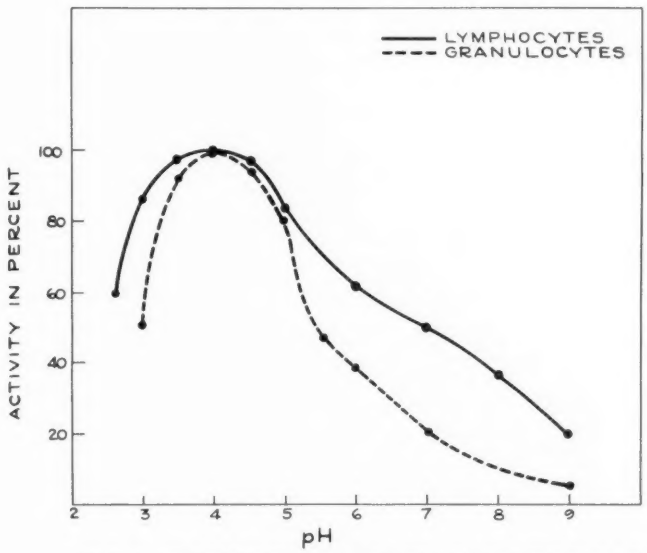

FIG. 1. EFFECT OF pH ON NUCLEOTIDASE ACTIVITY OF LEUKOCYTES

The variation in nucleotidase activity between granulocytes and lymphocytes is a clear demonstration of a biochemical difference in the two cell types. Such differences could have been predicted on the basis of variations in staining characteristics and in response to noxious agents.

Although the degree of reversibility of the leukocyte phosphatase activity is not known, Osgood and co-workers[14] have shown in tissue culture studies with leukocytes from leukemia patients that the incorporation of radioactive phosphorus into desoxyribonucleic acid is much greater in granulocytes than

in lymphocytes. Hence, apparently synthetic as well as degradative processes involving inorganic phosphorus and nucleic acid derivatives can proceed with greater rapidity in granulocytes.

In contrast to variations in nucleotidase activity between the leukocytes, we have found* that for other phosphatases, notably adenosine triphosphatase and pyrophosphatase, the activities of granulocytes and lymphocytes are similar. Since granulocytes are the cell types containing high nucleotidase activity, we have also made preliminary observations on this enzyme during P^{32} therapy and have found that the activity ratio of A-5-P to A-3-P is apparently changed.

SUMMARY

It has been found that the nucleotidase of leukocytes is an acid phosphatase with a pH optimum of 4.0. Evidence has been presented that the activity of this enzyme is not influenced by the nature of the ribose to phosphorus linkage in the nucleotide substrate. Nucleotidase activity measures ten times greater in the granulocyte than the lymphocyte.

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RENAL FUNCTION IN MULTIPLE MYELOMA

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THE sequence of development of the renal lesion in multiple myeloma was described by Bell[1] as a precipitation of Bence-Jones protein in the tubule, a resultant foreign body reaction, and finally the loss of the entire nephron unit. This concept fits the descriptions presented by earlier observers, and subsequently has been generally upheld and confirmed. However, many patients have had evidence of renal damage, yet Bence-Jones proteinuria was not demonstrated. Also, albuminuria has been noted frequently, and cannot be explained by tubule blockage alone. Finally, experiments in which Bence-Jones protein has been injected into animals for relatively short periods of time have resulted only in an occasional tubular obstruction.[2] There are several possible explanations for these discrepancies. First, the age range of patients with multiple myeloma makes possible the coexistence of other renal disease. Second, the clinical reports of albuminuria in the absence of Bence-Jones proteinuria are not always convincing. And, finally, the development of lesions, either toxic or obstructive, may require a longer period of time than has been used experimentally, or may involve some other complicating factor such as partial urinary obstruction.

Recently, Armstrong[3] has challenged the basic correctness of the tubule obstruction concept. He has shown, by performing renal clearance tests on patients with multiple myeloma, that the glomerular filtration rate, renal plasma flow, and tubular excretory maximum do not decrease proportionately, as would be expected if complete nephron units were destroyed. He observed that the glomerular filtration rate and renal plasma flow fell at a more rapid rate than did the tubular excretory mass. Similar studies in this laboratory have failed to confirm this finding, therefore the data are being presented below.

CASE MATERIAL AND METHODS

Studies were performed on 9 patients with multiple myeloma. Ten individuals without evidence of renal impairment were also studied for control purposes. The diagnosis was confirmed by bone marrow aspiration in all cases. Data pertaining to these individuals are summarized in Table I. Endogenous creatinine clearances were used to indicate glomerular filtration rate (GFR), and para-aminohippuric acid was used to determine renal plasma flow (RPF) and tubular excretory mass (Tm). The Brod and Sirota[4] modification of the Bonsnes and Taussky method was used for the determination of creatinine, and the method

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TABLE I. CLINICAL DATA ON PATIENTS WITH MULTIPLE MYELOMA

PATIENT	AGE	PROBABLE DURATION OF DISEASE	BLOOD PRESSURE	SERUM UREA NITROGEN MG./100 ML.	SERUM ALB./GLOB.	BENCE-JONES PROTEINURIA G PER DAY	ALBUMINURIA	DATE OF STUDY
D.	61	2½ yr.	140/80	23	3.2/5.4	3.5	0	6/ 9/49 1/20/50
E.	41	5	130/80	8	3.8/1.2	3.5	0	6/10/49 7/ 7/49
M.	55	2	135/80	16-30	3.0/4.1	10.8	+	7/14/49
W.	34	2 mo.	90/55	49 (NPN)	3.5/5.2	0	0	8/26/49
C.	62	2 yr.	150/95	17	3.3/5.5	+	+	12/13/49
F.	38	1	128/64	50-80	1.9/6.0*	18.0	+	12/16/49
B.	35	4	136/82		3.7/2.7	5.4	+	3/ 3/50
E. E.	70	4 mo.			4.8/1.4	0	0	5/ 3/50
W. E.	58	4 yr.	130/80	18	4.4/6.5	0	+	8/ 2/50

*Cryoglobulins present.

of Smith and his associates[5] was used for the determination of para-aminohippuric acid. In most experiments 3 periods were used to determine the renal plasma flow and 3 more to determine the tubular excretory mass. Tubular excretory mass was calculated on the basis of the concurrent filtration rates.

TABLE II. RENAL CLEARANCE DATA ON CONTROLS AND PATIENTS[1]

NAME	AGE	GFR_1	RPF	FF	GFR_2	TM	GFR_1/TM	GFR_2/TM	GFR_2/GFR_1
				Normal					
E. S.	24	131	766	0.17	118	109	1.20	1.08	0.90
K.	27	115	578	0.20	107	70	1.63	1.36	0.84
T.	27	103	513	0.20	98	69	1.49	1.42	0.95
C. S.	34	101	588	0.17	82	94	1.07	0.85	0.82
K. S.	34	103	566	0.18	89	109	0.95	0.82	0.86
M.	33	94	442	0.21	78	61	1.53	1.28	0.83
B.	39	89	370	0.24	76	100	0.90	0.76	0.85
F. B.	45	109	639	0.17	83	84	1.30	0.98	0.75
T. M.	44	72	439	0.16	57	93	0.77	0.62	0.80
G.	45	71	355	0.20	60	96	0.73	0.62	0.85
Mean	35.2	98.8	526	0.190	84.8	88.5	1.157	0.979	0.845
Standard deviation	7.8	18.4	128	0.025	19.2	16.4	0.324	0.296	0.055
				Multiple Myeloma					
E.*	41	146	785	0.18	117	124	1.17	0.94	0.80
B.	35	105	681	0.15	82	109	0.96	0.75	0.78
W. E.	58	81	345	0.23	73	79	1.02	0.92	0.90
M.	55	80	443	0.18	71	56	1.44	1.27	0.88
D.*	61	75	382	0.20	64	50	1.50	1.20	0.85
C.†	62	67	419	0.16	49	52	1.28	0.95	0.81
W.	34	50	258	0.19	49	35	1.40	1.38	0.99
E. E.	70	38	135	0.29	36	21	1.86	1.73	0.93
F.	38	21	122	0.17	19	17	1.27	1.16	0.91
Mean	50.5	73.7	397	0.194	62.2	60.3	1.333	1.144	0.872
Standard deviation	13.6	37.0	226	0.042	28.6	37.2	0.274	0.298	0.069

*Average of studies on two different dates.

†The only female in the group. Since determinations on this patient were so close to the mean, it was felt that separate tabulation was unnecessary.

GFR_1 is glomerular filtration rate as estimated from endogenous-creatinine clearance concurrently with measurement of PAH clearance at low-plasma levels.

GFR_2 is a similar measurement determined concurrently with the estimation of T_{mPAH}.

Values have been corrected to 1.73 square meters of body surface.

Oral temperatures averaged 98.2° F., with a range of 1° F. or less, and no corrections were applied.

RESULTS AND DISCUSSION

Table II presents the average renal clearance data for the 9 patients with multiple myeloma and the 10 normal controls. It can be seen that while there is evidence of renal impairment in most cases of multiple myeloma, the filtration fraction is normal except in one, indicating a parallel loss of glomerular and vascular tissue. The variations in the ratio between the filtration rate and the tubular excretory mass for para-aminohippurate excretion in multiple myeloma is not statistically different from the same ratio in the normal group. This suggests that the tubular excretory function also decreases at a rate proportionate to the decrease in vascular and glomerular function.

Three necropsies were obtained soon enough after the clearance studies to afford some anatomico-physiologic correlation. Examination of the kidneys of patient E. demonstrated minimal anatomical evidence of multiple myeloma, which was compatible with the absence of renal functional impairment. The tissues of patient D. showed marked atherosclerotic changes and moderate evidence of protein cast formation with secondary degenerative changes. The third patient, F., demonstrated a classic myeloma kidney with little evidence of any other type of renal lesion. It should be noted that the ratios in these 3 patients were not significantly different from each other. In E. E. the high filtration rate to tubular excretory maximum ratio was associated with a high filtration fraction. This suggests the possibility of a physiologic rather than an anatomic explanation for the elevated filtration rate.

A number of workers have shown that endogenous creatinine clearance is comparable to inulin clearance, but that there may be an error at clearances below 40 ml. per minute.[4] Camara and his co-workers[6] suggest that compensating physiologic and analytic errors tend to correct for the factor of tubular excretion of endogenous creatinine. The clearance of creatinine dropped at serum levels of para-aminohippurate high enough for Tm determinations, perhaps as a result of blockage of tubular excretion.[7] This fall was comparable in all control and myeloma patients, and suggests that the tubular excretory factor was essentially the same for all individuals in the series. The constancy of the filtration fraction and the constant decrease in the creatinine clearance upon para-aminohippurate loading is the basis of our belief that the creatinine clearances were useful for this study although their value for indicating absolute glomerular filtration rate may be questionable. It must be noted that the low values for glomerular filtration rate during the test for tubular excretory mass resulted in unusually high Tm values.

This study of renal function in multiple myeloma and that of Armstrong[3] include 24 patients. Of these only about one-half had sufficient renal impairment at the time of study to show a significant discrepancy from the normal values. The wide variation of results in so few patients makes mean values of doubtful significance, and may explain the different interpretation of results in the two reports. The parallel reduction in filtration rate, renal plasma flow, and tubular excretory mass seems consistent with observed anatomic changes.

CONCLUSIONS

Glomerular filtration rate, renal plasma flow, and tubular excretory mass were determined by endogenous creatinine and para-aminohippurate clearances in 9 patients with multiple myeloma and in 10 normal individuals. The means of these values were significantly lower in the patients with multiple myeloma. However, the filtration fractions, the ratios between the filtration rates and the tubular excretory maxima, and the ratios of the filtration rates before and after para-aminohippuric acid loading were the same in both groups. This suggests that the reduction of glomerular, vascular, and tubular function is parallel, and the result of destruction of entire nephron units.

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ABNORMALITIES IN THE EXCRETION OF WATER AND SODIUM IN "COMPENSATED" CIRRHOSIS OF THE LIVER

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PATIENTS with advanced cirrhosis of the liver in whom there is progressive edema and ascites manifest impairment in the renal excretion of sodium[1-5] and of administered water loads,[6-8] as well as reversal of the diurnal excretory rhythm of sodium and chloride.[9-11] These abnormalities have not been demonstrated in nonedematous cirrhotic patients.

Each of these abnormalities might be of discrete origin. Thus, retention of sodium has been related to increased production of adrenocortical steroids[4, 12] and that of water to increased activity of antidiuretic hormone (ADH).[6] Alternatively, they may be interrelated. For example, the same derangements which stimulate salt retention might also result in increased secretion of ADH. Moreover, since the ability to excrete water loads rapidly is decreased when sodium output is lowered[13-16] the cirrhotic, eliminating a virtually sodium-free urine, might exhibit impaired water excretion even in the absence of increased ADH activity.

The study reported here is aimed at evaluating the presence, interrelationship, or independence of these disturbances in cirrhotic patients not accumulating salt and water. In such circumstances, mechanisms which frequently coexist, might be found to be dissociated.

MATERIALS AND METHODS

Six patients with uncomplicated portal cirrhosis associated with prolonged excessive alcohol intake (Table I) and six healthy young male volunteers were selected for study. The diets were nutritionally adequate; that first supplied contained about 35 meq. of sodium daily; this was later supplemented with sodium chloride to provide about 225 meq. daily. A minimum of four days on each regimen preceded any studies. Water intake, except as indicated below, was not restricted.

None of the patients manifested subcutaneous edema; only one had minimal ascites at the onset of the study; patients 5 and 6 were losing latent edema, as indicated by weight losses of 5.6 and 5.2 kg., respectively, and high rates of sodium output during the twenty-five days of observation. Weight gains in patients 1 to 4 and controls ranged from 0.8 to 2.6 kg. on the higher sodium intake.

The observations made on each regimen concerned (1) renal excretion of sodium, (2) diuretic response to an ingested water load, and (3) the diurnal rhythm of sodium and potassium excretion. During each experiment the subject remained in bed after 8 P.M., took no food and drank no fluid except during the period of water loading. Collections were made of the urine passed between midnight and 6 A.M. and between 6 A.M. and 9:30 A.M. in three

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TABLE I. CLINICAL DATA IN PATIENTS WITH CIRRHOSIS

SUBJECT	AGE (YR.)	SEX	LIVER SIZE* (CM.)	ICTERUS INDEX	BSP† (PER CENT)	WATER TEST	SERUM SODIUM (MEQ./L.)
1 H. M.‡	59	M	4	< 5	15	Normal	139
2 P. G.	40	M	3	7	15	Abnormal	142
3 N. J.	41	F	3	7	23	Abnormal	140
						Normal§	
4 J. N.‖	49	M	5	20	54	Abnormal	141
5 T. D.	43	M	4	< 5	15	Normal	140
6 J. W.	43	M	2	< 5	15	Abnormal	140

*Centimeters below the right costal margin.

†Bromsulfalein—per cent retained in blood forty-five minutes after intravenous injection of 5 mg. per kilogram of body weight.

‡Had had ascites two years prior to study. No ascites for more than one year. No salt restriction for at least six months.

§Abnormal before study and normal after study.

‖Had minimal ascites at time of study.

of the six control subjects and all of the patients. At 9:30 A.M. each subject consumed 20 ml. of tap water per kilogram of body weight in a period of ten to forty minutes; this water load was sustained for two hours by the ingestion of enough water to maintain body weight constant after each voiding. Urine was collected every thirty minutes during the period of diuresis and then at hourly intervals until 3:30 P.M. Venous blood was obtained before water loading, at the height of the diuresis, and at the end of each study. The recumbent posture was maintained throughout, except when the subjects stood to void or to be weighed.

A standard Robinson-Power-Kepler water test[7] was performed just before and again at the conclusion of the series of studies in each patient.

Sodium and potassium were determined by means of an internal standard flame photometer. Chloride was determined by the method of Schales and Schales.[17] Creatinine in urine was determined by the method of Peters,[18] and in serum by that of Hare.[19]

RESULTS

The four patients in relatively static condition will be considered first. While on the salt-poor diet, their rates of sodium excretion were comparable in range to that of the normal subjects (Tables II and III). However, with high

TABLE II. AVERAGE RATES OF URINARY EXCRETION OF SODIUM AND POTASSIUM AT NIGHT AND MAXIMUM DAYTIME RATES OF EXCRETION OF SODIUM AND POTASSIUM (DURING AND AFTER WATER LOADING) WHILE ON LOW SALT AND HIGH SALT DIETS

	LOW SALT DIET				HIGH SALT DIET			
	SODIUM (MEQ./MIN.)		POTASSIUM (MEQ./MIN.)		SODIUM (MEQ./MIN.)		POTASSIUM (MEQ./MIN.)	
SUBJECT	NIGHT	DAY	NIGHT	DAY	NIGHT	DAY	NIGHT	DAY
Normal								
J. K.	0.001	0.011	0.029	0.114	0.126	0.350	0.039	0.089
W. S.	0.001	0.045	0.034	0.131	0.056	0.342	0.022	0.069
R. E.	0.001	0.015	--	--	0.200	0.312	0.036	0.133
Patient								
1 H. M.	0.017	0.074	0.055	0.122	0.151	0.275	0.030	0.139
2 P. G.	0.022	0.068	0.032	0.077	0.099	0.168	0.016	0.087
3 N. J.	0.034	0.053	0.056	0.083	0.183	0.109	0.048	0.071
4 J. N.	0.038	0.035	0.026	0.066	0.152	0.098	0.029	0.053
5 T. D.	0.024	0.127	0.040	0.161	0.188	0.360	0.058	0.098
6 J. W.	0.029	0.191	0.025	0.122	0.470	0.376	0.046	0.107

TABLE III. RATES OF WATER AND SODIUM EXCRETION DURING PERIOD OF MAXIMAL URINE FLOW AFTER WATER LOADING*

		LOW SALT DIET						HIGH SALT DIET					
				SODIUM		CREATININE				SODIUM		CREATININE	
	SUBJECT	BODY WEIGHT (KG.)	URINE FLOW (ML./MIN.)	(MEQ./MIN.)	(MEQ./L.)	CLEARANCE (ML./MIN.)	U/P	BODY WEIGHT (KG.)	URINE FLOW (ML./MIN.)	(MEQ./MIN.)	(MEQ./L.)	CLEARANCE (ML./MIN.)	U/P
Normal													
	A. D.	73.4	10.8	0.013	1.2	133	12.3	74.8	22.0	0.308	14.0	142	6.5
	R. V.	84.8	20.0	0.035	1.7	200	10.0	85.6	30.0	0.440	14.6	162	5.4
	R. B.	65.4	11.5	0.051	4.4	137	11.9	66.4	15.5	0.285	18.4	153	9.9
	J. K.	69.4	11.8	0.005	0.4	126	10.7	70.5	18.4	0.241	13.1	128	6.9
	W. S.	69.2	8.2	0.045	5.5	139	17.0†	70.0	18.5	0.342	18.5	136	7.3
	R. E.	74.5	9.6	0.012	1.3	--	--	77.1	17.5	0.304	17.3	182	10.3
Patient													
1	H. M.	104.0	7.7	0.074	9.6	133	17.2	105.0	9.8	0.218	22.2	136	13.8
2	P. G.	67.8	7.3	0.047	6.5	117	16.0	68.8	9.3	0.156	16.8	125	13.5
3	N. J.	67.4	5.3	0.053	10.0	121	22.8	69.0	6.9	0.109	15.9	109	15.8
4	J. N.	69.5	7.5	0.015	2.0	123	16.4	71.4	8.1	0.074	9.1	118	14.6
5	T. D.	105.0	8.5	0.104	12.2	217	25.5	101.2	16.0	0.360	22.5	195	12.2
6	J. W.	83.6	9.3	0.191	20.5	116	12.5	80.0	13.7	0.376	27.4	127	9.3

*Periods were sixty to ninety minutes.

†The high creatinine U/P ratio and marked delay in the onset of diuresis after water loading suggest incomplete suppression of ADH activity in this experiment in which the subject was anxious and apprehensive.

salt intakes three of the four attained maximal rates of sodium excretion which were considerably less than those observed in the normal subjects under similar dietary conditions (Tables II and III and Fig. 1).

At each level of salt intake the maximal rates of urine flow achieved were consistently below the range of values obtained in the normal subjects, so that the ratios of the concentration of creatinine in the urine to that in the plasma (U/P) were higher than in the control group. This abnormality in water excretion was more apparent during the high salt regimen (Table III, Fig. 1).

While taking the salt-poor diet, these patients exhibited nocturnal rates of sodium excretion which were higher than in any of the three subjects who served as controls; in two of these patients the nocturnal excretion rate approached or equalled the rate by day (Table II, patients 3 and 4). Frank reversal of the diurnal rhythm was evident in these two patients while they were on the high salt diet (Table II, Fig. 2).

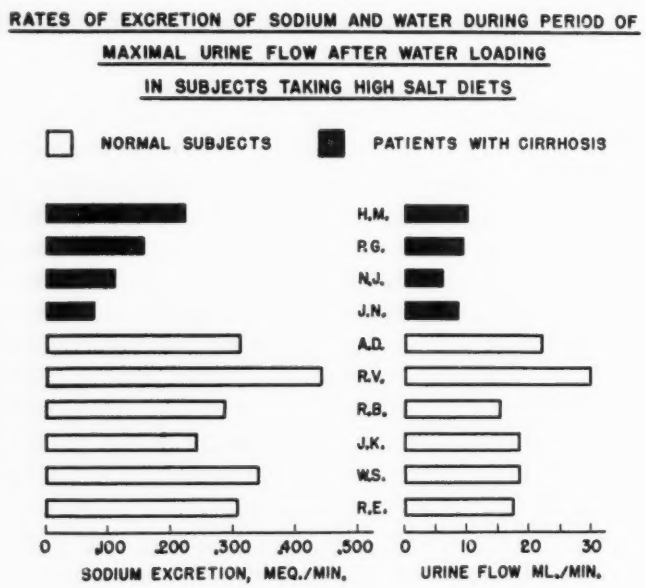

Fig. 1.

The two patients (5 and 6) who were losing latent edema during the period of study may now be considered. On the low-sodium diet both excreted larger amounts of sodium than any of the control subjects both by day and at night (Table II). In spite of this natriuresis, they failed, after water loading, to achieve urine flows greater than those of the controls (Table III). While on the high salt diet these two patients had maximal rates of sodium excretion in the morning that were within the range of the normal controls (Table II). In one the night rate of excretion exceeded that of the day.

The standard water test was abnormal in three of the patients with cirrhosis both before and after the period of study (Table I). A normal water

test was obtained in two cirrhotics and a third had an abnormal test before study and a normal one at the end of the experiments. No correlation between severity of disease and the results of the water test was apparent. This was also true in eight other cirrhotics not reported here. Of the two patients having a spontaneous diuresis, one had a normal water test and the other did not. There was no consistent relationship between the results of the water test and the diurnal rhythm of sodium excretion, nor between the results of the water test and the ability to excrete sodium and water on a high salt regimen.

All subjects exhibited a morning rise in potassium excretion on both the low salt and high salt diets (Table II). Since the diets were not constant in potassium content, comparison of excretion rates has little significance.

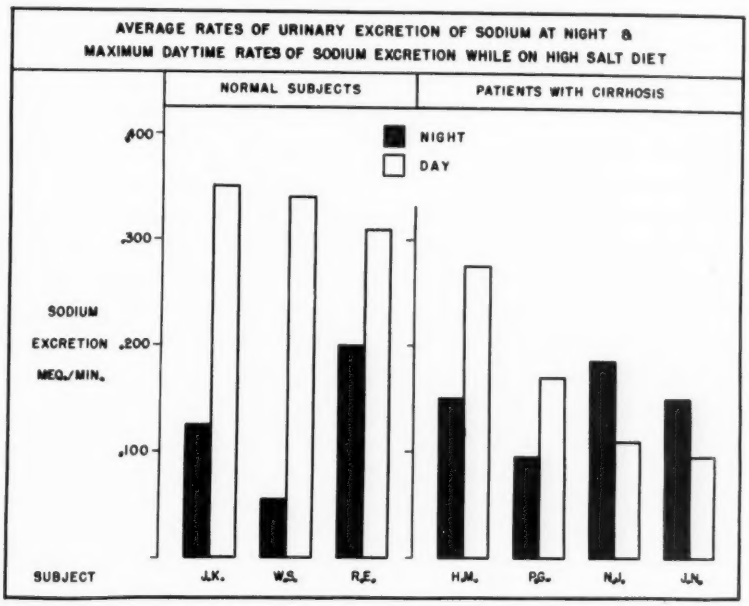

Fig. 2.

Certainly there were no consistent differences between the excretory pattern of the patients and the normal subjects. Of the two patients whose diurnal rhythm of sodium excretion was reversed, one appeared to have a normal excretory pattern for potassium, whereas in the other the nocturnal excretory rate seemed relatively high when compared with the morning rate.

DISCUSSION

Each of the four patients in relatively static condition, while taking a diet liberal in sodium, manifested some significant difference from the normal controls. All four had distinct impairment in the diuretic response to water loading, and in three, renal sodium excretion in the daytime was definitely diminished on the high salt regimen. Differences were less evident when the diet was poor in sodium. The two patients undergoing spontaneous diuresis achieved rates of sodium excretion comparable to those of the normal subjects. However during

the period of salt restriction, the maximal rates of urine flow and minimum creatinine U/P ratios after water loading in these two patients, indicated that their capacity to excrete water was no greater than those of the controls who were excreting much less sodium.

The number of cases we have studied is too small to permit conclusions concerning the incidence of abnormalities in the renal excretion of water and electrolytes in patients with cirrhosis without edema or ascites. The frequency with which such functional alterations were demonstrable in the patients we have studied suggests that their presence is not uncommon. Although Ralli observed that after water loading, nonedematous cirrhotic patients achieved maximal rates of urine flow and chloride excretion not significantly different from the normal, she did describe a sharper fall in minute urine volume after peak diuresis than was observed in the normal subjects.[8] It is difficult to compare her observations with those reported here since most of the data are presented as group averages.* Moreover, data concerning sodium excretion and variations in glomerular filtration[20] are not presented. Goodyer and his co-workers have reported that two nonedematous cirrhotic patients manifested a normal increase in renal sodium excretion after the intravenous administration of hypertonic saline.[5] As they point out, however, such studies provide different information from that afforded by observations concerning the manner of excretion of a large daily intake of sodium chloride with free access to water.

The observed decreased diuretic response to water loading in nonedematous cirrhotic patients may be considered in relation to the known effects of electrolyte excretion and antidiuretic hormone on rate of urine flow. The present study confirms previous observations that the maximal rate of urine flow in normal subjects in whom ADH activity is suppressed by water loading, varies directly with the concomitant rate of sodium excretion.[13-16] Therefore, in three of the cirrhotic patients taking the high-salt diet, the relatively low rates of sodium excretion, in part at least, account for the decreased diuretic response to water loading. This relationship does not preclude the coexistence of increased antidiuretic activity in these patients. The recognition of such antidiuretic activity may be difficult since even in the absence of ADH, the precise ratio of rate of urine flow to rate of sodium excretion is quite variable. Nevertheless certain observations strongly suggest the presence of increased antidiuretic activity in the patients studied. For example, the patient with minimal ascites attained a maximal rate of urine flow which was not significantly higher when he excreted 74 microequivalents of sodium per minute on the high-salt diet than when the sodium intake was restricted and its excretion was only 15 microequivalents per minute. Moreover, all but one of the normal subjects, even while excreting far less than 74 microequivalents of sodium per minute, were able to attain higher urine flows and lower U/P ratios for creatinine. Another patient while on the high salt diet excreted sodium at a rate which approached that of the normal controls, but excreted water at a far lower maximal rate with

*One of the patients in Ralli's "compensated" group who was having a spontaneous diuresis excreted water during the hour of peak flow at a rate almost 50 per cent higher than that of another patient in this group who was not undergoing diuresis. This pattern is not unlike the data reported here.

a minimal creatinine U/P ratio well above the range of the normal subjects. It may also be noted that while on the low-salt diet the two patients undergoing spontaneous diuresis had elevated rates of sodium excretion, but their rates of urine flow were no higher than those of the normal controls who excreted far less sodium. The presence of increased antidiuretic activity in cirrhotic patients without ascites is also indicated by the assays reported by Van Dyke, Ames, and Plough.[21] Thus the decreased diuretic response to ingested water in nonedematous cirrhotic patients may be related both to decreased natriuresis and increased antidiuretic activity.

The present study indicates that the diurnal rhythm of sodium excretion may be abnormal in the patient with "compensated" cirrhosis and in the cirrhotic patient undergoing diuresis as well as in patients with edema and ascites. Although the patients in the present study demonstrated abnormalities in the morning rate of sodium excretion on the high-salt diet, they did not gain more weight than the healthy control subjects. These observations are consistent with the fact that the total daily renal excretion of sodium chloride is within normal limits in cirrhotic patients free of ascites.[22] It may therefore be inferred that cumulative sodium retention was obviated as a consequence of the excretion of more salt at other times of the day. Indeed, two of the subjects whose condition was relatively static exhibited frank reversal of their diurnal rhythm of sodium excretion, as did one of the patients who was having a spontaneous diuresis. In these patients a large moiety of the twenty-four hour output of sodium was excreted at night.

The simple water test merely compares the night urine flow following a day of moderate salt restriction with the rate of urine excretion after administration of a water load the next morning. This test may be abnormal while diuresis is occurring because the nocturnal rate of urine flow is high. The test may be normal in patients who demonstrate distinct impairment in water excretion while on a diet containing liberal amounts of sodium chloride. It is apparent, therefore, that in some patients with cirrhosis the water test alone cannot suffice as a measure of the capacity to excrete water.

Since the nonedematous cirrhotic may excrete sodium and water relatively normally on a low salt diet and not on a high salt diet, it is probable that the accumulation of edema and ascites could have been induced in some of the patients with prolonged intake of sufficient quantities of sodium chloride. The converse, that the development of edema may be prevented or postponed by salt restriction, may apply. It is conceivable, therefore, that moderate salt restriction in those cirrhotics without edema who demonstrate abnormalities in the renal excretion of sodium and water may add to the benefits of other therapeutic measures by delaying the onset of abnormal fluid retention.

SUMMARY AND CONCLUSIONS

In three patients with "compensated" cirrhosis of the liver and one with minimal ascites, abnormalities were demonstrable which included a decreased diuretic response to water loading, a diminished maximal rate of sodium excretion when dietary intake was large, and alteration in the diurnal rhythm of sodium excretion.

The observations in two of these four patients strongly suggest the persistence of antidiuretic activity after a water load had been established.

Two patients with cirrhosis of the liver without apparent edema or ascites, who were losing latent edema, also manifested differences from control subjects with regard to water diuresis and sodium excretion.

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BROMSULFALEIN CLEARANCE: A QUANTITATIVE CLINICAL TEST OF LIVER FUNCTION

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INTRODUCTION

IT HAS long been accepted that the liver is one of the most complex organs of the body, and studies of its functions have continually challenged the clinical investigator. No single test of liver function is specific or universally accepted as the most reliable. One of the more generally satisfactory and clinically approved tests is the bromsulfalein excretion test. The purpose of this report is to describe a modification of this test which enables the clinician to obtain a quantitative assessment of liver function.

METHODS

Male patients were selected from the wards of a large hospital population and tested under standard conditions that consisted of (1) a fasting state for ten hours, (2) the absence of fever, (3) the absence of jaundice, and (4) the maintenance of the supine position for the duration of the test.

A dye-free blood sample is withdrawn prior to the intravenous injection of five milligrams of bromsulfalein per kilogram of body weight. The injection requires about thirty seconds, and the mean injection time is recorded as zero time. Carefully timed blood samples are obtained thereafter at intervals of five, ten, fifteen, twenty, thirty, and forty-five minutes. The mean sampling time is recorded to the nearest second with a stop watch. Each blood sample (1.5 to 2.0 milliliters) is transferred to a small test tube for mixing with oxalate crystals, using due care to avoid hemolysis. The oxalated blood is then transferred to mercury-calibrated Wintrobe tubes and centrifuged at 2,500 r.p.m. (15 centimeter radius) for thirty minutes. A measured volume of the supernatant plasma is transferred to a Beckman pyrex absorption cell (one centimeter light path) containing 2.0 ml. of a 0.1N solution of sodium hydroxide. The bromsulfalein dye concentration is calculated from the optical density of the solution at 575 mμ using a Beckman Model B spectrophotometer. A reference blank consisting only of a 0.1N solution of sodium hydroxide is used in order to correct for the optical density of dye-free plasma, since the concentration of plasma in each absorption cell is not always identical.

The logarithm of the plasma dye concentration is plotted against time in minutes (Fig. 1). The intercept of the extrapolated line with the ordinate, when time equals zero, provides an estimate of the initial dye concentration. Since the quantity of injected dye is known, the volume into which this dye is distributed can be calculated. Although this volume really represents the bromsulfalein compartment, the fact that bromsulfalein becomes bound to plasma protein[1] permits the assumption that this estimated volume is

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identical with the plasma volume. Furthermore, it has been demonstrated that the slope, k, of the semi-log plot in Fig. 1 is proportional to the fractional clearance (c/v),[2, 3] i.e., $c/v = 2.3 \times \frac{(\log P_1 - \log P_2)}{t_2 - t_1}$. By multiplying fractional clearance (c/v) by the estimated plasma volume (v) we obtain clearance (c) expressed in milliliters of plasma per minute. Fig. 1 illustrates a sample calculation from the data obtained in the case of T. C., a control subject. These calculations may be simplified by plotting the data directly on semi-log graph paper. In Fig. 2 it is evident that both control and test data confirm the previously reported observations that bromsulfalein disappearance is exponential.

The procedure described in this report is being adapted for general clinical laboratory use employing a Coleman Junior spectrophotometer and 5 ml. blood samples. The calibrated Wintrobe tubes were used in this study merely as a convenience for handling small blood volume samples. As long as the exact volume of plasma diluted with the sodium hydroxide solution is known, one may employ any suitable volumetric container.

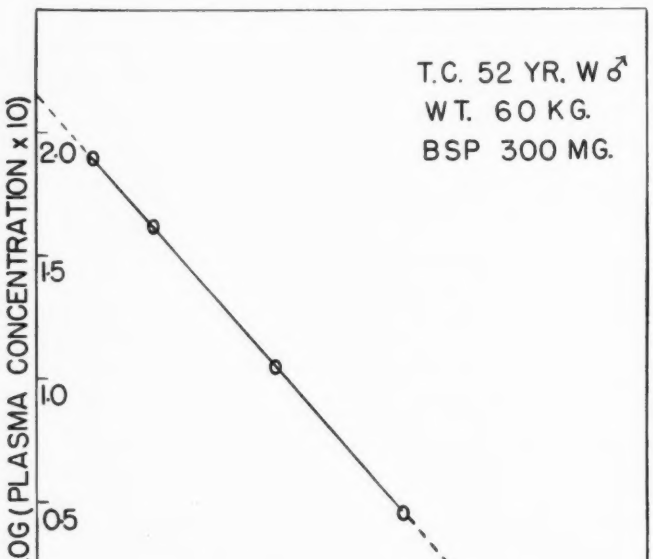

Fig. 1.—Sample calculation:

Antilog 1.16 = 14.4 mg./100 ml.
v = (300/14.4) × 100 = 2082 ml.
k = 2.16/38 = .0568
c/v = 2.3 × .0568 = .1308
c = .1308 × 2082 = 275 ml./min.
c/kg. = 275/60 = 4.54 ml./min.

RESULTS

The data obtained by this test appear in Table I. Expressing these results on a per kilogram of body weight basis, the control subjects had a mean clearance value of 5.28 ± 1.00 ml. of plasma per minute. Application of the "t" test shows that these mean values are significantly different ($p < 0.001$).

It has been shown that plasma volume measurements in man, based upon the Evans Blue dye dilution technique, yield a mean value of 44.0 ml. of plasma per kilogram of body weight. Accepting this as an empirical reference, the mean plasma volume of all the subjects included in this study (except R. H.) was 2,873 ml. ± 382 ml. The mean estimated plasma volume for the same group determined by the bromsulfalein dye dilution method was 2,589 ml. ± 565 ml. Application of the "t" test in this instance shows that these values are probably not significantly different ($p = 0.20$).

DISCUSSION

Most clinicians are familiar with the original bromsulfalein excretion test[4] in which the per cent of dye retention is calculated forty-five minutes after the intravenous injection of 5 mg. of dye per kilogram of body weight.

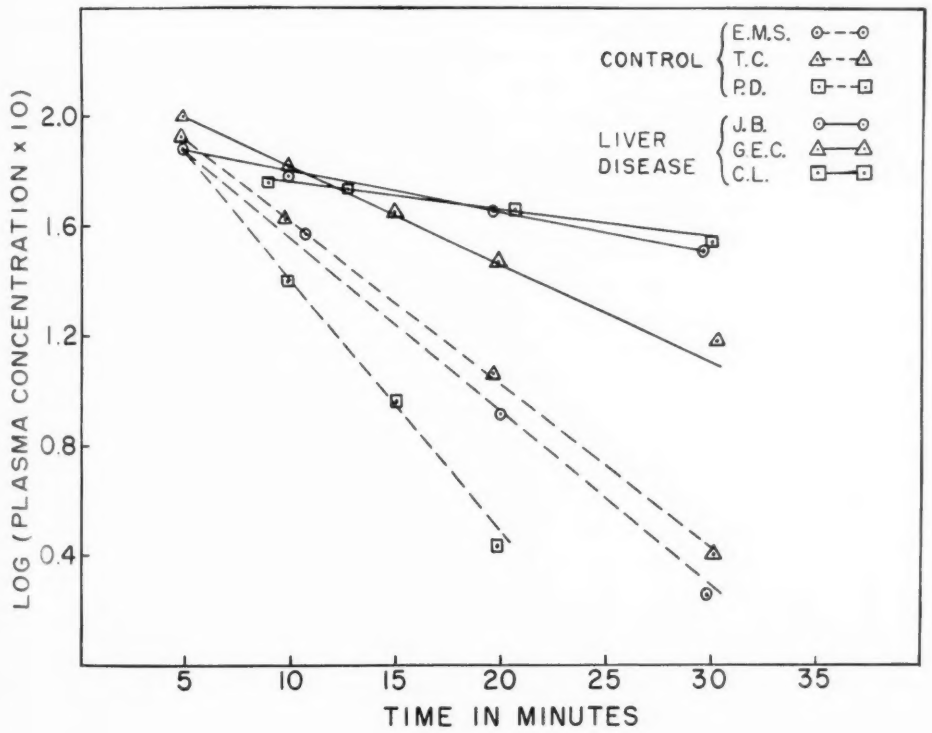

Fig. 2.

Subsequently, Ingelfinger and his co-workers[5] published an excellent report describing the behavior of bromsulfalein disappearance from the blood stream of healthy subjects as well as of persons having hepatic disease. This investigation was extended by Mendeloff and associates,[6] who showed that repeated bromsulfalein tests under comparable conditions yielded reproducible results, one normal subject having been tested five times. More recently, this test was

modified by Lavers and collaborators,[7] who followed a procedure similar to the one reported here. They designated the slope, k, of the semi-log plot as the "clearance coefficient." Their patients with known liver disease had k values less than 0.037, while 90 per cent of their controls had values greater than 0.037, with an average of 0.082. Interestingly enough, in a study of thirty normal young persons, Mateer and associates[8] made serial blood estimations of residual dye content at five-minute intervals up to the forty-five-minute period. From these data it was possible for us to compute the values of the slopes, k, for the semi-log plots of dye content against time. These values ranged from 0.045 to 0.080, with a mean of 0.073. In our controls the k values ranged from 0.057 to 0.084, with an average of 0.067. Those with liver disease had an average k value of 0.022, with a range of 0.010 to 0.038. The excellent agreement of these three studies is noteworthy.

Finally, the resolution of the "clearance coefficient" into its component functions, as described by Lewis,[2, 3] made it possible to design the bromsulfalein clearance method presented in this report.

In some instances we were able to confirm the observations of Lavers and associates[7] that the semi-log plot does not always remain linear beyond the twenty-minute interval. In some of these cases the determined blood levels were higher than the predicted values. Lavers and co-workers suggested that these high blood levels in patients with liver disease were due to an inability of the liver to excrete bromsulfalein into the bile as fast as it was being cleared from the blood. By the twenty-minute period a retrograde block occurred within the liver which in turn slowed the clearance from the blood stream.

TABLE I

SUBJECT	DIAGNOSIS	WT. IN KG.	K	C/V	V	C	C/KG.
			Control Group				
E. M. S.	Gastritis	71	0.0629	0.145	2248	325	4.58
J. W. S.	Duodenal ulcer	67	0.0632	0.145	3260	473	7.06
T. C.	Prolapsed gastric mucosa	60	0.0568	0.131	2082	275	4.54
R. R.	Gastric ulcer	60	0.0573	0.132	2030	268	4.46
J. R.	Duodenal ulcer	65	0.0766	0.176	2088	368	5.66
P. D.	Gastric ulcer	81	0.0840	0.193	2248	434	5.36
		Mean	0.0667	0.154	--	357	5.28
		S. D.					1.00
			Liver Disease Group				
J. B.	Chronic hepatitis	70	0.0129	0.0296	3680	109	1.55
G. E. C.	Cirrhosis	76.4	0.0384	0.0884	2345	207	2.71
C. H. L.	Cirrhosis	57.3	0.0382	0.0879	2468	217	3.79
R. H.	Cirrhosis	70	0.0085	0.0195	5180*	101	1.45
C. L.	Cirrhosis	57	0.0263	0.0605	2850	173	3.03
I. V. B.	Cirrhosis	53.6	0.0102	0.0235	3185	75	1.40
		Mean	0.0224	0.0516	--	147	2.32
		S. D.					1.00

k = Clearance coefficient.
c/v = Fractional clearance.
v = Estimated plasma volume in milliliters.
c = Clearance in milliliters of plasma per minute.
*Subject R. H. had about 2 liters of ascitic fluid.

However, the recent report of Lorber and Shay,[9] demonstrating the enterohepatic circulation of bromsulfalein, suggests that the absorption of bromsulfalein from the intestine may be responsible for these unpredicted higher blood levels occasionally noted at the thirty- and forty-five-minute periods. Since the data necessary for the estimation of clearance by our method can be obtained within twenty minutes, there appears to be no justification for a forty-five-minute test.

Some objections to the measurement of dye retention by a single estimation at the forty-five-minute period are that (1) an error may be introduced by the absorption of bromsulfalein from the upper gastrointestinal tract, (2) it is difficult to make reliable colorimetric measurements with low dye concentrations, and (3) the influence of changes in plasma volume and hepatic blood flow cannot be assessed. The advantages of the bromsulfalein clearance test as presented in this report are: (1) the test may be completed within twenty minutes, (2) an estimate of the plasma volume can be made, (3) the clearance, expressed as milliliters of plasma per minute per kilogram of body weight, is a dynamic concept consistent with our knowledge of the kinetics of bromsulfalein disappearance from the blood stream, and (4) this expression of clearance may be compared with similar data in readily comprehensible terms. Although it is not possible to make direct measurements of the hepatic blood flow by this approach, it should be possible to determine the relative effects of changes in hepatic blood flow upon clearance without the necessity of catheterizing the hepatic vein.[10, 11]

SUMMARY

A simple clinical test for the quantitative measurement of bromsulfalein clearance has been described. The semi-log plot of dye disappearance is extrapolated to zero time to obtain the volume of fluid into which the bromsulfalein is distributed. This has been assumed to be the plasma volume. The fractional clearance (c/v) is obtained from the slope of this semi-log plot. Clearance is then obtained as the product of the fractional clearance and the plasma volume.

In control subjects the mean value for clearance was 5.28 ± 1.00 ml. per minute per kilogram. Subjects with liver disease in the absence of fever and jaundice had a mean value of 2.32 ± 1.00 ml. per minute per kilogram. It has been shown that this difference in means is significant. Moreover, it is particularly significant that the determined clearance coefficients obtained by three independent investigations showed excellent agreement.

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THE PORTAL CIRCULATION TIME IN CIRRHOSIS OF THE LIVER FOLLOWING PORTACAVAL ANASTOMOSIS

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PORTACAVAL anastomosis is gaining wide recognition in the treatment of portal hypertension. During the past year, we have had the opportunity of observing selected patients with cirrhosis of the liver in whom this operation was performed. In these cases we have studied the portal circulation time, as originally described by Newman and Cohen,[1] before and after surgery. The data obtained suggest that this test may be useful in evaluating the degree of patency of the shunt following operation. They also confirm the validity of certain of the assumptions made by the original authors.

METHOD

The method for the determination of portal circulation time is exactly as described by Newman and Cohen. The procedure is as follows: Two 100 c.c. syringes are supported in a metal frame, and connected by a Y-tube to a rubber tube (Fig. 1). Three cubic centimeters of ethyl ether are introduced into the tubing, which is then clamped with a hemostat. The tubing is immersed in hot water until the liquid ether vaporizes to 200 c.c., and then connected to a rectal tube which has been inserted in the rectum 10 inches beyond the anus. The hemostat is released and the vapor rapidly instilled. The time between instillation and the detection of ether on the patient's breath is the portal circulation time. We have found it necessary to perform the test after the rectum has been emptied to prevent absorption of vapor by the stool.

RESULTS

Our data in normal persons and in patients without hepatic, pulmonary, or circulatory disease agree closely with those of Newman and Cohen. In ten such subjects the portal circulation time was between twenty and twenty-five seconds. In twenty patients with Laennec's cirrhosis (proved by biopsy) having portal hypertension and esophageal varices, the portal circulation exceeded twenty-five seconds. Portacaval anastomosis was performed in seven of these patients. The results of these tests are presented in Table I. It will be seen that in each case there was a significant decrease in the portal circulation time following operation.

DISCUSSION

The measurement of portal circulation time as devised by Newman and Cohen is a simple and direct test. The assumptions described by these authors are as follows:

1. No ether vapor is absorbed via the inferior hemorrhoidal vein (which would gain access to the systemic circulation and by-pass the liver).

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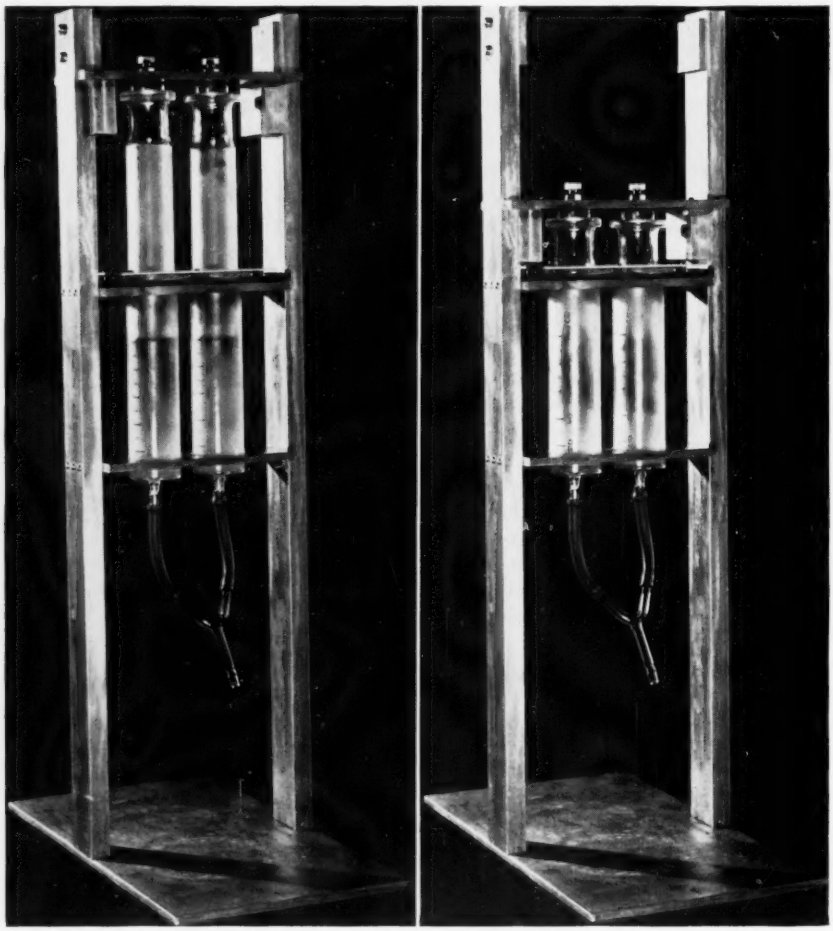
2. The rate of flow through the portal system is inversely proportional to the portal pressure.

3. Intestinal absorption of ether is rapid and relatively unchanged in different subjects. (This seems likely since the range of values in normals is relatively narrow.)



A. *B.*

Fig. 1.—Apparatus for portal circulation time. *A*, Before, and *B*, after instillation of ether vapor.

The insertion of the rectal tube 10 inches above the anus places the tip well above the lower rectum. If ether vapor were absorbed in significant amounts via the inferior hemorrhoidal vein, one would expect circulation times in the same range as those obtained following shunt (unless portacaval anastomosis increases the permeability of the intestinal mucosa to ether). In cirrhosis with portal hypertension, where the hemorrhoidal plexus is usually

TABLE I. PORTAL CIRCULATION TIME AFTER PORTACAVAL ANASTOMOSIS

CASE	DIAGNOSIS	PORTAL CIRCULATION TIME (IN SECONDS)		DIFFERENCE IN SECONDS
		Before shunt	*After shunt*	
1	Laennec's cirrhosis	32	22	10
2	Laennec's cirrhosis	32	21	11
3	Laennec's cirrhosis	38	24	14
4	Laennec's cirrhosis	42	18	24
5	Laennec's cirrhosis	26	12	14
6	Laennec's cirrhosis	26	16	10
7	Laennec's cirrhosis	34	13	21

more prominent than normal, one would expect an even greater contribution by the inferior hemorrhoidal system. It is in such cases, however, that the portal circulation time is prolonged. Hence it does not seem likely that the systemic venous drainage of the lower rectum interferes with the test. It is also of considerable interest that the portal circulation time in patients with a colostomy is the same as if the tube were inserted into the rectum.[2]

The data following portacaval shunt suggest that the second assumption is also valid. In each of our cases the portal pressure measured after the anastomosis was markedly decreased, and in each case this drop in pressure was associated with a decrease in circulation time through the portal bed.

Although the preoperative time was only slightly prolonged in cases five and six (both small females), the low postoperative values suggest that the normal for these patients is less than the observed normal.

In patients with congestive failure or prolonged systemic circulation time of any cause, prolonged portal circulation time does not necessarily indicate portal hypertension. It seems desirable, therefore, to perform the systemic and pulmonary circulation times if such complications are present, as suggested by the original authors.[2]

Finally, it is suggested that the lowered circulation time observed following shunt may be an index of the patency of the anastomosis. We have tested two patients repeatedly over a five-month period. In one, the portal circulation time has remained low and esophagoscopy has confirmed the efficacy of the shunt by demonstrating the continued absence of varices. In the other, the only patient with a splenorenal shunt, the portal circulation time was slightly elevated three months after operation and moderately elevated (thirty-two seconds) at six months. Esophagoscopy has revealed a recurrence of varices in this patient with a gradual increase in the pressure measured in these veins.

In a paper read before the Society of Medicine and Surgery of Sao Paulo by de Almeida (abstracted in the *Journal of the American Medical Association* in correspondence from Brazil),[3] it was also suggested that the portal circulation time might be of value in determining the patency of a portacaval shunt. The procedure used differs somewhat from that described by Newman and Cohen, but the principles involved are similar.

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SUMMARY

The portal circulation time of Newman and Cohen has been measured before and after portacaval anastomosis in seven patients with cirrhosis of the liver. In each instance there was a marked decrease in the observed time. The test seems to be useful in determining the patency of the shunt following operation.

Four additional patients with cirrhosis of the liver and esophageal varices have had postcaval shunts, bringing the total to eleven cases studied. The results in these patients were similar to those listed in Table I.

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METABOLISM OF IRON

II. Intravenous Iron Tolerance Tests in Laennec's Cirrhosis

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IN A previous study, intravenous iron tolerance tests were performed on normal subjects and on patients with hemochromatosis.[1] Normal ranges were established, and a characteristic deviation from this normal pattern was noted in hemochromatosis. There is evidence that therapeutic phlebotomy may be of specific value in the treatment of hemochromatosis.[2, 3] Early diagnosis is therefore imperative. The major problem in the diagnosis of this disease rests in its differentiation from Laennec's cirrhosis with or without diabetes.

Intravenous iron tolerance tests were performed on patients with Laennec's cirrhosis in order to evaluate this test as a differential diagnostic aid. An attempt was made to correlate the characteristics of the test with the various clinical and laboratory data observed. In addition, a small number of patients with acute hepatitis and with diabetes and hepatomegaly were also studied.

The intravenous iron tolerance test permits measurement of the fasting serum iron level (SeFe) as the first specimen, the total iron binding capacity (TIBC) as that specimen drawn five minutes after the injection of ferric ammonium citrate, and the 120-minute serum iron level. The ratio SeFe/TIBC represents the degree of saturation of the iron binding protein. The increase (INC) from the fasting serum iron level (SeFe) to the total iron binding capacity (TIBC) is the unsaturation iron binding capacity. The decrease (DEC) in the serum iron level from the five-minute to the 120-minute specimen is apparently a function of the tissue uptake of serum iron. In normal subjects there is a fairly constant relationship between the increase (INC) and the decrease (DEC), conveniently expressed as the DEC/INC ratio.

METHODS

Seventeen intravenous iron tolerance tests were done on sixteen patients with Laennec's cirrhosis proved by liver biopsy. Except for one patient (Case 22), all biopsies were performed within two weeks of the intravenous iron tolerance tests. Six of this group had manifest diabetes. Five tests were performed in four patients classified as probable portal cirrhosis on clinical grounds alone. Three of these, each with a negative skin biopsy, had mild diabetes. The remaining patients in this group did not have diabetes and refused skin and liver biopsies.

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TABLE I. INTRAVENOUS IRON TOLERANCE TESTS AND CLINICAL DATA IN LAENNEC'S CIRRHOSIS

CASE	SeFe (γ%)	TIBC (γ%)	120′ (γ%)	SATURATION (%)	DEC/INC RATIO	SDI	CEPHALIN FLOCCULATION	THYMOL TURBIDITY (UNITS)	BILIRUBIN (MG. %)	ALBUMIN / GLOBULIN (GM. %)	ALKALINE PHOSPHATASE (S. UNITS)	PROTHROMBIN TIME AND CONTROL (SECONDS)	BSP (% RETENTION)	HEMOGLOBIN (GM. %)	HEMATOCRIT (%)	DIABETES (1-3+)	LIVER BIOPSY	SKIN BIOPSY	G.I. BLEEDING (1-4+)	LIVER*	SPLEEN*	ASCITES	EDEMA	SPIDERS	LIVER PALMS	HEMORRHOIDS
1.	186	284	254	66	30/98 0.31	97	1+	19.8	2.4	3.5/4.4	8.4	21.1 (17.0)	24	17.8		2+		N	1+	3fb	2fb	+	+	0	0	0
2.	157	314	268	50	46/157 0.29	79																				
3.	64	209	168	31	45/145 0.31	62	4+		2.1	3.9/3.2	11.8			11.7	31	3+	CA	NA	4+	4fb	2fb	+	+	+	+	+
4.	63	180	159	35	21/127 0.17	52																				
5.	109	225	177	48	48/116 0.41	89	1+		1.9	3.0/3.6	9.8	24 (16.3)		12.1	42	1+	C	N	3+	5fb	2fb	+	+	+	+	+
6.	51	176	132	29	44/125 0.35	64	2+	8.2	12.8	3.9/2.5	7.2	25.1 (16)		9.3	33	1+		N	1+	4fb	0	0	0	+	+	0
7.	37	269	174	14	95/232 0.41	55	1+	4.3	2.4	3.4/3.7			4	10.6	32	0			2+	2fb	2fb	+	+	+	+	+
8.	86	205	129	42	76/119 0.64	106	4+	10.6	4.3	2.4/3.6	5.4	23.2 (15.3)		13.5		0	CA	NA	0	5fb	4fb	+	0	0	0	+
9.	92	313	229	29	84/221 0.38	67	0	2.0	3.1	4.6/4.0	6.8	19.2 (15.3)		11.9		2+	C		0	3fb	0	+	+	0	0	+
10.	45	242	141	19	101/197 0.51	70	3+		0.8		6.3	15.1 (14.3)	9	11.4	36	1+		N	4+	3fb	2fb	+	+	0	0	+

11.	124	332	280	37	52/208 0.25	62	3+	2.7	0.4	4.2/3.0		14.9 (15.2)	8	16		0	C		0	2[fb]	0	0	0	0	0	0
12.	127	288	257	44	31/161 0.19	63	0		3.0	4.7/4.5	8.5		24	15.2	47	0	C		0	2[fb]	0	+	+	+	+	0
13.	124	288	151	43	136/164 0.84	127	1+	7.3	0.8	4.1/2.5	7.1	15.4 (14.0)		13	41	1+	C		0	5[fb]	0	+	+	0	+	0
14.	32	175	131	18	44/143 0.31	49	0		0.4	4.2/2.5	1.6		3	9	32	3+	C	N	0	3[fb]	0	0	0	0	0	
15.	165	292	256	56	36/127 0.28	84	4+	17.3	3.3	2.2/4.2	7.3		34	11.4	35	0	C		0	3[fb]	0	+	+	+	+	
16.	171	285	261	60	24/114 0.21	81	2+	10.0	0.7	3.5/4.3	9.8	19.4 (16.6)		16	47	3+	C		0	3[fb]	0	0	0	+	+	0
17.	162	311	296	52	51/149 0.34	86	0	7.7		4.0/3.5		16.4 (15)	15	14.6		0	C		0	5[fb]	0	0	0	0	0	+
18.	118	340	304	35	36/222 0.16	51	0		6.7	4.1/4.2	13.5		22	10.4		0	C		2+	4[fb]	0	+	0	0	0	
19.	133	269	258	49	11/136 0.08	57	2+	27.3	1.4	3.2/3.2	7.3	16 (14)	28	11.4		0	C		0	5[fb]	0	0	0	+	+	0
20.	57	288	131	20	157/231 0.68	88	3+	3.0	1.9	3.8/2.8	11.1	17.0 (15.0)	12	15.3		0	C		0	4[fb]	0	0	+	0	0	
21.	220	317	273	69	44/97 0.45	114	4+	31.8	2.2	2.5/4.0	9.4	19.3 (16.0)	35	15.3	49	1+	C			3[fb]	1[fb]	0	0	+	+	+
Average	111	267	210	40	0.36	76																				
22.	187	207	181	90	26/20 1.30	220	0	3.4	2.2	3.1/3.4	10.2			13.4	40	0	C		1+	3[fb]	0	0	0	0	0	0

SeFe, fasting serum iron; TIBC, total iron binding capacity (5 minute specimen); saturation, SeFe/TIBC × 100; INC, increase (TIBC-SeFe); DEC, decrease (TIBC – 120 feet); SDI, $\frac{\text{per cent saturation}}{100}$ + DEC/INC; H, hepatitis; N, normal; C, cirrhosis; A, autopsy; O, known negative results or findings; blank, results or findings not known.

*As finger-breadths below costal margin.

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Four studies were done on three patients with acute infectious hepatitis. In two of the three the diagnosis was established by liver biopsy. Two patients with diabetes, hepatomegaly, normal liver biopsies, and pre-existing cardiac failure were also studied.

The intravenous iron tolerance tests were done in the manner previously described.[1] Serum iron determinations were performed by slight modifications of the method of Balle.[4] The normal ranges for the laboratory studies utilized were: cephalin flocculation, 0 to 2 plus in forty-eight hours;[5] thymol turbidity, 0 to 5 units;[6] serum bilirubin, 0.7 to 1.0 mg. per cent; albumin/globulin ratio, 4.5 to 5.5 Gm. per cent/1.5 to 3.0 Gm. per cent;[8] alkaline phosphatase, 2.2 to 8.6 Schinowara units;[9] bromsulfalein test, less than 5 per cent retention in the serum after forty-five minutes.[10] The plasma prothrombin time was determined by the Link Shapiro modification of the Quick one-step method.[11]

The severity of diabetes was classified from one to three plus. One plus signifies that the diabetes was revealed only by a diabetic glucose tolerance curve. Two plus indicates the presence of a fasting hyperglycemia, occasional glycosuria, and treatment with minimal amounts or no insulin. Three plus signifies the requirement of moderate to large amounts of insulin daily. Bleeding, either as melena or hematemesis, was classified according to severity from one to four plus. Liver biopsies were performed with a Vim-Silverman needle using either the lateral intercostal or the anterior subcostal approach.

RESULTS

All results are presented in Tables I, III, and IV.

Laennec's Cirrhosis.—For reasons to be presented later, the values of the test performed on Case 22 are not included in the range and average figures listed below. The SeFe values ranged from 32 μg to 220 μg per 100 ml. of serum, with a mean of 111 μg per cent. The TIBC values ranged from 175 μg to 340 μg per cent, and averaged 267 μg per cent. The average saturation of the serum iron binding protein was 40 per cent, with a range of 14 to 69 per cent. The serum iron concentrations decreased from the peak TIBC values to an average of 210 μg per cent at 120 minutes. The latter ranged from 129 to 304 μg per cent. The DEC/INC ratio varied from 0.08 to 0.84, averaging 0.36. It should be noted that all the DEC/INC ratios are less than 1.0, and that only three of the twenty-one are greater than 0.51.

Acute Hepatitis.—The patient in Case 30 expired of fulminating hepatitis within one week of performance of the intravenous iron tolerance test. The patient in Case 31 recovered from his acute illness and was restudied two months after the first test. At that time the SeFe was 172 μg per cent, the TIBC 330 μg per cent, the saturation 52 per cent, the 120 minute level 318 μg per cent and the DEC/INC ratio 0.08. The last patient, Case 32, was studied three days after the onset of icterus.

Diabetes with Hepatomegaly and Normal Liver Biopsy.—As will be noted in the discussion, the test results presented in Table IV are within the limits of normal.

DISCUSSION

The average SeFe in normal male subjects is 146 μg per cent, with a range of 74 to 186 μg per cent.[1] The TIBC values range from 253 to 388 μg per cent, and average 341 μg per cent. The average saturation of the serum iron binding protein is normally 41 per cent, with a range of 28 to 58 per cent. The 120 minute values average 289 μg per cent, varying from 163 to 335 μg per cent. The normal DEC/INC ratio ranges from 0.10 to 0.50, averaging 0.26.

As compared with these normal figures, intravenous iron tolerance tests in cirrhosis reveal: (1) a slightly lower average SeFe value with a greater range; (2) an appreciably lower average TIBC level with a spread beginning below but falling partially within the normal range; (3) a normal average saturation with a slightly increased range; (4) a slightly higher average DEC/INC ratio reflecting an elevated upper limit of the range.

There is considerable overlap between the results of the iron tolerance test in normal subjects and patients with portal cirrhosis. The test is therefore in no way diagnostic for the latter. This disease is frequently associated with an iron tolerance test normal in every respect.

Seven of the twenty-one tests have SeFe values below the lowest limit of normal, and in six of these seven there is an associated anemia with hematocrit values below 37 per cent. In five of these seven patients active gastrointestinal bleeding was noted at the time of this study. Three of the remaining patients have demonstrable anemias with normal SeFe values, but in each of these the bromsulfalein test reveals marked retention. In no case with anemia and a low SeFe is the bromsulfalein retention greater than 9 per cent. The only case (Case 21) of fasting hyperferremia is associated with a normal hematocrit and the highest bromsulfalein retention in the series. It is therefore apparent that anemia tends to be associated with hypoferremia and bromsulfalein retention with elevation of SeFe values. We have failed to demonstrate any correlation between the SeFe levels and the other clinical and laboratory factors noted in Table I.

Since uncomplicated blood loss or iron deficiency anemia may be associated with hypoferremia and a high TIBC value,[14] the question arises as to what effect gastrointestinal bleeding would have on the intravenous iron tolerance test in the cirrhotic patient. That anemia and blood loss complicating portal cirrhosis tend to be associated with hypoferremia is obvious from the above data. This hypoferremia may be modified by the tendency towards elevation of the SeFe values in cases with more acute and severe hepatic dysfunction (i.e., as measured by the BSP test or as noted in acute hepatitis[13]). However, no case of portal cirrhosis with anemia, hypoferremia, or demonstrable gastrointestinal bleeding revealed an abnormally elevated TIBC value. This is evidently related to the primary tendency of any case of portal cirrhosis to low TIBC values.

In eight of the tests performed, the TIBC values are below the normal range. In no case is this five-minute level above normal. There is no apparent correlation between the TIBC level and any of the clinical or laboratory data studied. Despite this fact, it is our impression that lower TIBC values are found in the more severely ill patients. The remaining test values, i.e., 120 minute specimen, saturation, and DEC/INC ratio, also fail to correlate with any of the clinical or laboratory data presented in Table I, except in so far as they are reflections of those changes in the SeFe levels noted above.

The intravenous iron tolerance tests in hemochromatosis, as presented in Table II, have the following characteristics: (1) a normal or high SeFe (120

to 297 μg per cent, averaging 213 μg per cent); (2) a normal or low TIBC (164 to 335 μg per cent, averaging 246 μg per cent); (3) a high degree of saturation of the iron binding protein (63 to 99 per cent, averaging 94 per cent); and (4) a high DEC/INC ratio (0.59-5.33, averaging 2.09).[1]

A comparison of Tables I and II demonstrates the effectiveness of the intravenous iron tolerance test as a diagnostic aid in differentiating Laennec's cirrhosis from hemochromatosis. Although the average SeFe in hemochromatosis is much higher than that in portal cirrhosis, there is nevertheless a significant overlap in the distribution of individual values in the two groups. The SeFe value alone is therefore of little diagnostic significance. On the other hand, it should be noted that in no instance was hypoferremia (below 70 μg per cent) found in hemochromatosis. This is in marked contradistinction to the findings in cirrhosis, and occurred despite considerable blood loss in several of the patients with hemochromatosis. Fifty per cent of the SeFe levels in the cirrhosis group are below the lowest SeFe figure noted in any patient with hemochromatosis (120 μg per cent). One-third of all the patients with cirrhosis demonstrated true hypoferremia. It should be emphasized that a normal or high SeFe value may occur in either of these two diseases. Range and average TIBC values are also comparable in both diseases.

The saturation and DEC/INC ratio appear to be the most important factors in the use of the test for differentiating the above diseases. Although in general the values in hemochromatosis tend to be much higher, both factors exhibit small but definite areas of overlap. The saturation overlap extends from 63 to 69 per cent, into which range fall two cases of cirrhosis and one of hemochromatosis. The DEC/INC ratio overlap extends from 0.59 to 0.84,

TABLE II. INTRAVENOUS IRON TOLERANCE TESTS IN PATIENTS WITH HEMOCHROMATOSIS

CASE	SeFe (γ%)	TIBC (γ%)	120′ (γ%)	SATURATION (%)	INC (γ%)	DEC (γ%)	DEC/INC RATIO	SDI
23								
8/21/50	257	294	272	87	37	22	0.59	146
10/27/50	297	326	326	91	29	0	---	---
24								
9/12/50	196	222	124	88	26	98	3.77	465
9/14/50	133	212	114	63	79	98	1.24	187
11/16/50	201	222	167	91	21	55	2.62	353
25								
10/ 2/50	286	304	260	94	18	36	2.00	294
10/ 9/50	290	301	255	96	11	46	4.18	514
26								
10/20/50	204	207	199	99	3	16	5.33	632
11/ 8/50	197	207	187	95	10	20	2.00	295
27								
4/ 1/51	273	335	281	82	62	54	0.87	169
28								
5/17/51	153	195	164	78	42	31	0.74	152
5/26/51	120	164	131	73	44	33	0.75	148
29								
5/19/51	168	206	171	82	38	35	0.92	174
Average values	213	246	204	94	32	42	2.09	303

with three cases of cirrhosis and three of hemochromatosis coming within these limits. In other words, of the thirty-four iron tolerance tests performed on patients with cirrhosis and hemochromatosis, nine have one of these two factors falling in an indeterminate range. In no case do both factors fall within the overlap areas. In those cases of cirrhosis in which one factor lies within the indeterminate area, the other diagnostic factor always falls below the lowest limit of its overlap. Conversely, in hemochromatosis when one factor falls within the indeterminate range, the other invariably exceeds its upper limit of overlap.

In the hemochromatosis group, Case 23 has a DEC/INC ratio of 0.59, a figure within the DEC/INC overlap range. The saturation however, is 87 per cent, exceeding the upper limit of overlap for saturation. In the cirrhosis series, Case 21 has a saturation of 69 per cent, which forms the upper limit of the overlap range. The DEC/INC ratio in this case is 0.45, which falls below the DEC/INC indeterminate area. Therefore, in a test in which either the saturation of DEC/INC ratio falls within an indeterminate range, proper interpretation depends upon the relationship of the other factor to its area of overlap. The use of this principle afforded the correct diagnosis in all nine cases in which one factor or the other fell within the indeterminate range. This concept can be expressed mathematically by the simple addition of the saturation and DEC/INC values to yield a third factor, hereafter termed SDI. The SDI (per cent saturation/100 + DEC/INC) is below 1.35 in all cases of cirrhosis and above 1.35 in every patient with hemochromatosis.

The test values in patient 22 are included in Table I, but were purposely omitted from the calculation of average and range figures. Unfortunately, the liver biopsy revealing Laennec's cirrhosis could not be performed until one year after the intravenous iron test was done. The test results in this case are indistinguishable from typical hemochromatosis, and it is possible that this represents a true exception to the diagnostic criteria presented above. It is impossible to state whether or not this similarity to hemochromatosis is related to a superimposed hepatitis present at the time the iron tolerance test was done.

TABLE III. INTRAVENOUS IRON TOLERANCE TESTS IN PATIENTS WITH HEPATITIS

CASE	SeFe (γ%)	TIBC (γ%)	120′ (γ%)	SATURATION (%)	INC (γ%)	DEC (γ%)	DEC/INC RATIO	SDI
30	138	156	120	88	18	36	2.00	288
31	213	333	297	64	120	36	0.30	94
32	252	400	337	63	148	63	0.43	106

Table III presents the test results in three patients with acute hepatitis. Each has a high degree of saturation, and one of the three reveals an abnormally elevated DEC/INC ratio. It is therefore evident that hepatitis can simulate hemochromatosis insofar as the intravenous iron test is concerned. In two of the cases the SDI is below 1.35, and it is these two that reveal the typical abnormalities previously reported in hepatitis, namely a high SeFe,

with a normal TIBC.[12, 13] The third patient (Case 30), with test results identical to those seen in hemochromatosis, was an acutely ill man who expired within one week of the performance of the iron tolerance test. In this patient the high saturation was not due to the usually elevated SeFe but to an abnormally low TIBC. These results do not detract from the value of the intravenous iron tolerance test as a diagnostic aid for hemochromatosis, since the clinical differentiation of this disease from hepatitis is rarely difficult. As mentioned in the results, a repeat iron tolerance test in patient 31 during the recovery period revealed a return to normal of all values. Because of the small number of patients studied, no definitive statement can be made at this time concerning the characteristics of the intravenous iron tolerance test in acute hepatitis.

Since diabetes associated with hepatomegaly occasionally offers difficulty in differentiation from hemochromatosis, two such patients were studied. As presented in Table IV, the results of these two tests are completely normal.

TABLE IV. INTRAVENOUS IRON TOLERANCE TESTS IN PATIENTS WITH DIABETES AND HEPATOMEGALY

CASE	SeFe (γ%)	TIBC (γ%)	120′ (γ%)	SATURATION (%)	INC (γ%)	DEC (γ%)	DEC/INC RATIO	SDI (%)
33	87	267	235	33	180	32	0.18	51
34	87	314	247	28	227	67	0.30	58

Although other causes of hemosiderosis, such as untreated pernicious anemia, Cooley's anemia, and any anemia treated with blood transfusions but without blood loss may yield iron tolerance test results indistinguishable from those of hemochromatosis, the clinical differentiation of these diseases offers no problem. Laennec's cirrhosis remains the primary difficulty in the diagnosis of endogenous hemochromatosis. We feel that the intravenous iron tolerance test is of great value in making this differentiation. It is hoped that by the use of this test earlier diagnosis will be possible, thereby permitting initiation of therapy prior to the development of irreversible damage to the parenchymatous organs of the body.

SUMMARY

Twenty-eight intravenous iron tolerance tests were performed in patients with Laennec's cirrhosis, acute infectious hepatitis, and diabetes with hepatomegaly. Variations from the normal pattern and their possible significance were discussed.

The use of the intravenous iron tolerance test as a diagnostic aid in the differentiation of hemochromatosis from Laennec's cirrhosis was presented.

ADDENDUM

Since the completion of this study, Houston and Thompson (Houston, J. C. and Thompson, R. H. S.: Quart. J. Med. **21**: 215-224, 1952) have reported fasting serum iron and total iron binding capacity values in seven patients with hemochromatosis and one with diabetes and portal cirrhosis. Their results agree closely with those reported herein.

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DISTURBANCE OF VITAMIN B_6 METABOLISM IN PREGNANCY

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IT HAS been well established that a deficiency in the vitamin B_6 complex will alter the intermediary metabolism of tryptophane and lead to the excretion of the abnormal metabolite xanthurenic acid (4-8 dihydroxyquinoline-2-carboxylic acid) in the urine. Xanthurenic acid (XA) appears in the urine of various species, including the rat, under varying nutritional conditions, provided a tryptophane-containing protein is included in the experimental diet.[1] In the human being, however, even in severe vitamin B_6 deficiency induced by dietary means and the simultaneous administration of desoxypyridoxine which acts as a vitamin B_6 antimetabolite, XA will not be excreted unless large amounts of the pure amino acid are ingested.[2] Thus, it has been shown that human subjects made vitamin B_6 deficient by dietary means[3] or by the administration of desoxypyridoxine[4] excrete significant amounts of XA in the urine following the oral administration of 10 Gm. of dl-tryptophane. Sprince and his co-workers[5] fed tryptophane to normal women and women in the last trimester of pregnancy. Pregnancy in these women was either uncomplicated or complicated by toxemia or other diseases. Total XA excretion was not measured quantitatively, since only the concentration in the morning specimens before and following the test on several days was determined. Sprince and his co-workers found a significantly higher concentration of XA in the urines of women whose pregnancies were complicated by toxemia or other diseases than in those in which the pregnancy was apparently uneventful and normal nonpregnant control subjects.

In the present investigation the total excretion of XA was determined following the ingestion of 10 Gm. of tryptophane in normal subjects, patients with miscellaneous diseases, and in pregnant women with and without toxemia. The XA excretion was tested in various stages of pregnancy, and the influence of the administration of pyridoxine hydrochloride upon the excretion of the abnormal metabolite was examined.

MATERIAL AND METHOD

XA excretion was determined quantitatively by a modification of the method of Rosen and his co-workers.[6] In this modification, use is made of the excellent buffering capacity of tris(hydroxymethyl) aminomethane-maleate and of a precalibrated curve for the colorimetric quantitative determination of the green iron-xanthurenic acid compound which is formed after the addition of ferric ammonium sulfate.[7] Urine collection was started for a twenty-four hour period preceding the test. Ten grams of dl-tryptophane (Nutritional Biochemical Corp., Cleveland, Ohio) were then given in a glass of orange juice, and urine was collected for two twenty-four hour periods. In the latter part of the study only the first twenty-four hour specimens following the test meal were collected, since in only one instance was excretion of XA noted on the second day. In about 100 tests the urines were examined for the presence of XA preceding the test. The character-

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istic green color was never observed after addition of the reagent. However, small readings were obtained on the colorimeter due to the presence of interfering substances which had a brownish tinge. As can be seen from the tables, amounts of interfering substances in twenty-four hours varied between 0 and 23 mg., with an average of 5 milligrams.

Sarett and Goldsmith[8] reported that following the administration of dl-tryptophane, these chromogenic substances interfere seriously with the quantitative determination of urinary XA. This, however, was not found when l-tryptophane was used. Glazer and co-workers[4] were unable to confirm these observations, however, since patients with desoxypyridoxine-induced vitamin B_6 deficiency excreted the same amount of XA following the administration of either 10 Gm. of dl-tryptophane or 5 Gm. of l-tryptophane. Apparently only the l-isomer gives rise to XA. Glazer and co-workers also found that the use of ferric ammonium sulfate instead of ferric chloride for the development of the green iron xanthurenic acid complex minimized the interference of chromogenic substances in the quantitative test.

The administration of tryptophane was followed by severe diarrhea in only one case. Other than a transient feeling of slight nausea and occasional sleepiness, no untoward reactions were observed.

RESULTS

(A) *Normal Subjects.*—In 10 normal subjects, 6 women and 4 men between the ages of 20 to 50 years, the excretion of XA varied from 4 to 30 mg., with an average of 17 mg. (Table I and Fig. 1).

(B) *Patients With Miscellaneous Diseases.*—The tryptophane load test was performed on 22 patients who suffered from various diseases. As can be seen from Table II, this group included individuals with advanced carcinoma and apparent malnutrition, as well as patients with marked cirrhosis of the liver. Several of the cirrhotic patients had a history of severe alcoholism with reduced food intake. The excretion of XA in these patients varied between 0 and 74 mg., with an average of 20 milligrams. It can be noted from the table that in general the patients with apparent malnutrition showed the greater XA excretions. However, the highest value of 74 mg. was observed in a Puerto Rican man who had lobar pneumonia. No obvious history of reduced food intake could be obtained from this patient.

(C) *Pregnancy.*—The test was performed on 24 women one to two days after delivery of a full-term infant. Fourteen of these women had had uncom-

TABLE I. NORMAL CONTROL SUBJECTS

NO.	NAME AND AGE	SEX	EXCRETION BEFORE TRYPTOPHANE		EXCRETION AFTER TRYPTOPHANE	
			URINE IN 24 HR. (ML.)	INTERFERING SUBSTANCES IN 24 HR. (MG.)	URINE IN 24 HR. (ML.)	XA* IN 24 HR. (MG.)
1	J. T. 20	M	700	10	1,150	16
2	A. G. 27	M	850	4	1,000	4
3	G. W. 50	F	1,050	4	950	27
4	R. S. 28	F	1,050	7	850	15
5	H. M. 35	F	450	3	820	14
6	T. R. 31	F	250	3	1,280	24
7	B. W. 36	F	550	7	1,500	18
8	E. M. 36	F	1,250	21	1,400	30
9	M. W. 46	M	1,200	6	1,900	9
10	A. B. 31	M	1,330	6	1,100	15
Average				7		17

*The determination of XA includes interfering substances.

TABLE II. PATIENTS WITH VARIOUS DISEASES

NO.	NAME AND AGE	SEX	DIAGNOSIS	EXCRETION BEFORE TRYPTOPHANE		EXCRETION AFTER TRYPTOPHANE	
				URINE IN 24 HR. (ML.)	INTERFERING SUBSTANCES IN 24 HR. (MG.)	URINE IN 24 HR. (ML.)	XA IN 24 HR. (MG.)
1	L. K. 22	F	Infectious hepatitis	1,150	5	1,050	9
2	V. M. 55	F	Hypertensive heart disease	950	0	610	1
3	K. D. 61	M	Advanced liver cirrhosis, severe alcoholism	300	3	1,030	36
4	G. V. 74	F	Gastric ulcer, malnutrition	700	3	1,290	11
5	T. B. 40	M	Advanced liver cirrhosis, severe alcoholism	1,820	23	2,370	23
6	R. S. 64	F	Obstructive jaundice, malnutrition	764	8	1,350	41
7	M. L. 62	M	Carcinoma of lung, malnutrition	1,500	4	1,350	49
8	M. H. 37	F	Liver cirrhosis, alcoholism	2,000	7	850	11
9	W. F. 63	M	Hypertension	2,250	0	2,250	0
10	H. P. 42	M	Paroxysmal tachycardia	330	6	700	7
11	F. P. 43	M	Lobar pneumonia	3,700	0	4,000	74
12	J. C. 40	M	Myocardial infarction, duodenal ulcer	1,650	4	1,500	20
13	J. K. 70	M	Virus pneumonia, malnutrition	900	2	800	36
14	D. R. 40	M	Virus pneumonia	2,000	0	300	1
15	M. S. 48	F	Advanced cholangitic cirrhosis	850	7	500	14
16	A. S. 41	M	Advanced carcinoma of panereas	2,900	0	3,800	50
17	S. D. 68	M	Liver cirrhosis, alcoholism	850	1	450	6
18	B. J. 70	M	Obstructive jaundice	450	0	420	16
19	C. G. 46	M	Coronary heart disease	1,200	8	1,200	8
20	F. A. 59	F	Hypertensive heart disease	1,100	4	1,500	17
21	C. R. 71	F	Diabetes, nutritional anemia	2,600	0	2,400	20
22	P. M. 43	F	Advanced cirrhosis of liver, alcoholism	700	3	1,900	5
Average					4		20

plicated pregnancies, while 10 had shown signs of toxemia. All of these parturients had ingested a normal diet in apparently sufficient amounts. Some of the toxemic patients had been on a low Na diet for intermittent periods. This diet, however, allowed for ample nutrition, since it is normally rich in the vitamin B_6 complex. As can be seen from Table III, in the women who had undergone a normal pregnancy there was found an XA excretion which varied between 62 and 324, with an average of 191 milligrams. In the 10 cases of toxemia the excretion values varied from 94 to 666 mg., with an average of 314 mg. (Table IV). However, there was no correlation between the degree of toxemia and the amount of XA excretion, since several cases with severe involvement showed comparatively small amounts of excretion.

After it had been established that women one to two days post partum showed a significant increase in XA excretion, an investigation to determine

the time of onset of this abnormality of the tryptophane metabolism was instituted. As can be seen from Table V, a significant increase in XA excretion occurred in the twelfth to fourteenth week of pregnancy.

It is well known that certain substances are not present or occur only as traces in the urine of nonpregnant women which are found in significant quantities during gestation. Thus it has been known for many years that histidine is excreted early in pregnancy in comparatively large amounts.[9]

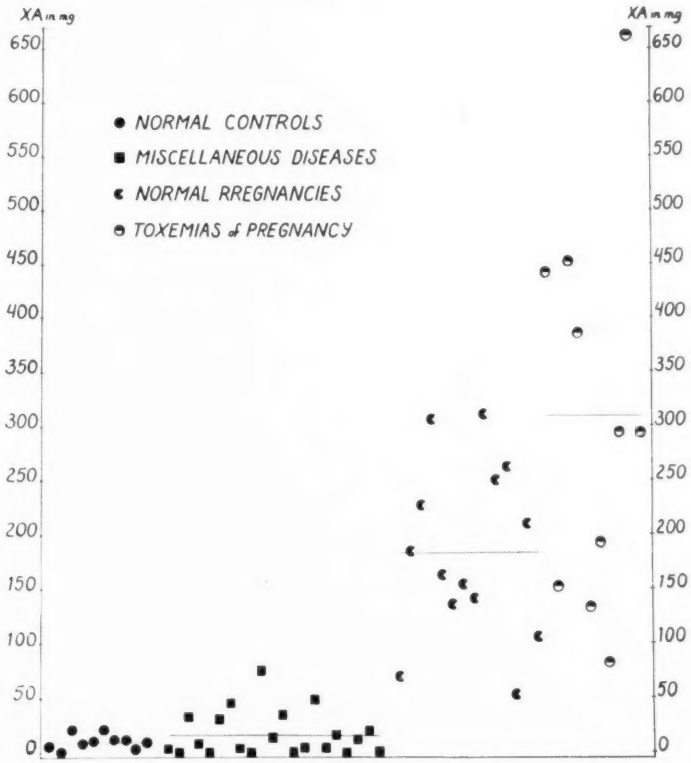

Fig. 1.—The urinary excretion of XA following a test dose of 10 Gm. of tryptophane in normal controls, patients with miscellaneous diseases, and in full-term pregnant women with and without toxemia.

More recently a similar increase in the urinary excretion of other amino acids has been detected.[10] The cause of this phenomenon is not completely understood. Changes in renal threshold as well as alteration in adrenal function influencing intermediary metabolism may be causative factors.[11] It was therefore considered necessary to rule out the possibility that the excretion of increased amounts of XA in pregnancy might not be due to a relative vitamin B_6 deficiency, but might rather represent another example of the phenomenon discussed above.

In order to clarify this point, 5 ambulatory patients in various stages of pregnancy were first tested for XA excretion and then treated with pyridoxine hydrochloride. Considerable variation of XA excretion may occur occasionally when the same pregnant individual is tested repeatedly without any vitamin

TABLE III. NORMAL PREGNANT WOMEN AT TERM

NO.	NAME AND AGE	EXCRETION BEFORE TRYPTOPHANE: URINE IN 24 HR. (ML.)	EXCRETION BEFORE TRYPTOPHANE: INTERFERING SUBSTANCES IN 24 HR. (MG.)	EXCRETION AFTER TRYPTOPHANE: URINE IN 24 HR. (ML.)	EXCRETION AFTER TRYPTOPHANE: XA* IN 24 HR. (MG.)
1	I. G. 28	1,500	11	1,000	80
2	Mrs. G. 25	1,220	16	725	200
3	D. Z. 20	650	6	1,250	233
4	J. D. 16	430	3	1,550	310
5	A. P. 30	2,400	6	1,150	169
6	M. G. 30	1,950	0	1,000	135
7	A. I. 20	1,250	2	1,000	156
8	M. D. 36	1,200	2	1,800	144
9	R. M. 24	1,700	12	1,620	324
10	Mrs. R. 25	1,100	8	740	259
11	C. G. 27	1,300	11	1,100	275
12	R. Z. 27	1,100	8	1,250	63
13	J. G. 18	950	5	900	217
14	J. Gr. 27	1,800	5	3,750	113
Average			6		191

*The determination of XA includes interfering substances.

B_6 supplement, as may be noted in Case 10 and Case 15 in Table V. In every instance the administration of pyridoxine hydrochloride led to marked diminution of the XA excretion (Fig. 2). Although in these preliminary experiments no attempt was made to establish the minimal dose necessary to suppress XA excretion, it can be seen that a substantial decrease of XA occurred after only three daily treatments with 25 mg. of pyridoxine hydrochloride. The additional administration for another three days reduced the excretion level to that observed in normal nonpregnant controls. In all instances in which enough time was allowed to elapse after the treatment had been discontinued, a marked increase in XA excretion following the test dose of tryptophane was again observed.

TABLE IV. PREGNANT WOMEN WITH TOXEMIA AT TERM

NO.	NAME AND AGE	EXCRETION BEFORE TRYPTOPHANE: URINE IN 24 HR. (ML.)	EXCRETION BEFORE TRYPTOPHANE: INTERFERING SUBSTANCES IN 24 HR. (MG.)	EXCRETION AFTER TRYPTOPHANE: URINE IN 24 HR. (ML.)	EXCRETION AFTER TRYPTOPHANE: XA* IN 24 HR. (MG.)
1	V. M. 30	760	10	1,350	162
2	J. W. 33	2,260	4	2,550	459
3	B. M. 20	390	1	1,875	388
4	S. K. 29	1,260	2	1,200	136
5	R. B. 23	1,150	1	1,270	195
6	L. B. 25	1,150	10	800	94
7	E. W. 42	2,000	5	1,500	300
8	H. P. 27	950	3	1,120	666
9	C. S. 23	800	6	1,450	300
10	D. S.	1,550	0	1,000	442
Average			4		314

*The determination of XA includes interfering substances.

DISCUSSION

Many surveys have been conducted in order to determine the occurrence of nutritional deficiencies in pregnancy.[12-16] In population groups in which food and vitamin intake are insufficient, clinical signs of certain vitamin deficiencies may be observed in the last trimester of pregnancy.[12, 17] Little is known about the requirement of the vitamin B_6 complex by the normal nongravid individual. Regarding requirements during pregnancy, contradictory statements are to be found in the literature. Vilter and his co-workers[12] did not feel that a deficiency of vitamin B_6 was an important factor. Hobson,[13] on the other hand, in a survey from England, found evidence for a deficiency

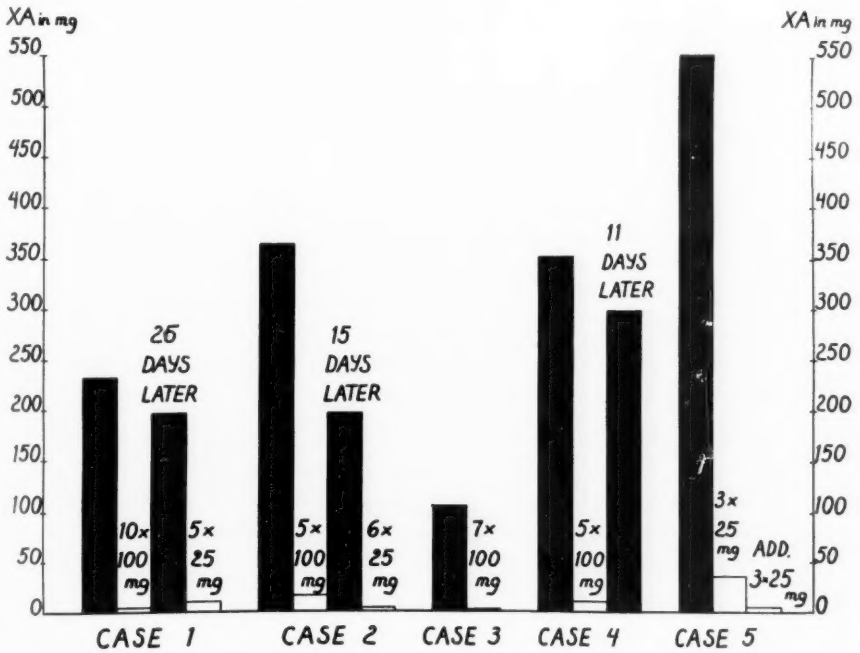

Fig. 2.—The urinary excretion of XA following a test dose of 10 Gm. of tryptophane in five pregnant women before and after the administration of pyridoxine hydrochloride. The black columns represent the excretion values before or at stated periods of time after discontinuation of the drug. The white columns represent excretion values immediately following the administration of various amounts of pyridoxine hydrochloride.

of both nicotinic acid and vitamin B_6 in the diet of pregnant women. Clinical manifestations of a deficiency in the vitamin B_6 complex apparently do not occur in human beings under natural conditions, although pyridoxine was described as having a curative effect on certain symptoms such as insomnia, irritability, and difficulty in walking in patients with nutritional deficiencies who had been treated previously with other members of the vitamin B complex.[18] Under experimental conditions, however, in two infants on a vitamin B_6 deficient diet[17] and in adults on such a diet supplemented with desoxypyridoxine,[2] clinical manifestations have been elicited which resemble those seen in experimental animals. The occurrence of seborrhea-like skin

TABLE V. WOMEN AT VARIOUS STAGES OF NORMAL PREGNANCY

NO.	NAME AND AGE	WEEK OF PREGNANCY	EXCRETION BEFORE TRYPTOPHANE		EXCRETION AFTER TRYPTOPHANE	
			URINE IN 24 HR. (ML.)	INTERFERING SUBSTANCES IN 24 HR. (MG.)	URINE IN 24 HR. (ML.)	XA* IN 24 HR. (MG.)
1	M. L. 22	8	1,200	6	1,350	9
2	D. S. 24	10	1,400	0	1,400	10
3	A. B. 29	11	1,100	4	1,150	31
4	J. H. 36	12	1,600	7	1,600	22
5	C. M. 28	12	1,850	5	2,000	156
6	C. D. 25	13	2,200	6	1,700	20
7	M. M. 28	14	1,450	4	1,500	107
8	C. C. 27	16	1,150	4	1,200	148
9	C. D. 35	17	870	5	750	245
10	A. N. 24	17	1,100	9	1,100	89
	A. N. 24	21	1,100	9	1,300	50
11	A. C. 32	17	1,200	6	2,000	372
12	C. C. 29	18	1,150	2	1,430	356
13	M. W. 40	25	1,300	0	1,350	135
14	B. M. 31	30	1,200	10	1,550	118
15	M. B. 17	33	750	3	600	84
	M. B. 17	35	750	3	850	555
16	E. S. 22	34	1,250	1	1,800	235

*The determination of XA includes interfering substances.

lesions, hypochromic, anemia, and epileptiform convulsions was noted. Although symptoms of this kind apparently do not occur in pregnant women, the evidence for the existence of a biochemically demonstrable vitamin B_6 insufficiency in pregnancy appears quite conclusive.

The nature of this disturbance is not clear at the present time. It could well be that in the pregnant state there occurs an abnormality in the metabolism of vitamin B_6 which leads to this increased production of XA. The possibility, however, that the growing fetus drains the maternal source of vitamin B_6 early in pregnancy must be considered. The vitamin B_6 complex was shown to be essential for the growing fetus by Cravens and Snell.[20] These investigators injected 500 to 1,000 mg. of desoxypyridoxine into eggs prior to the start of the incubation period, with a resulting mortality of 100 per cent. This inhibition was prevented by simultaneous injections of vitamin B_6. The same dose of desoxypyridoxine injected after four or more days proved nontoxic.

In view of the fact that elevation of blood pressure is the most important sign of toxemia in pregnancy, the observation that rats on a vitamin B_6 deficient diet develop a considerable rise in blood pressure is of some interest. Within a few days after the administration of pyridoxine the blood pressure of these rats reverts to normal.[21] The possible significance of the biochemically demonstrable disturbance in the metabolism of vitamin B_6 deficiency which occurs in pregnancy will have to be evaluated by carefully controlled studies. Experiments to determine the minimal amount of vitamin B_6 necessary to suppress the abnormal excretion of XA following the administration of tryptophane and thus to establish the maintenance dose necessary to supply the vitamin B_6 requirement of the pregnant women are in progress.

SUMMARY

After a test dose of 10 Gm. of tryptophane, only small amounts of XA are found in the urine of normal subjects, as well as patients with various diseases. Considerably larger amounts of this abnormal metabolite are excreted in pregnant women. The rise of XA excretion starts at the end of the first trimester. Following the administration of pyridoxine, the abnormally high excretion of XA is reduced to normal levels. Thus, evidence is presented for the presence of a biochemically demonstrable disturbance in the metabolism of vitamin B_6 during pregnancy.

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BRONCHOSPIROMETRY

V. Differential Residual Volume Determination

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INTRODUCTION

EVALUATION of pulmonary insufficiency requires information concerning the volume remaining in the lungs at the end of forced expiration—the residual volume. The technique for its determination, its normal value, and its alterations as a result of disease have received much attention. Of even greater clinical significance is the volume of gas remaining in the lungs at the end of a quiet expiration—the functional residual capacity. If either of these volumes is known, and the vital capacity and its subdivisions are determined by spirometry, the total lung capacity can be calculated. Clinically significant ratios can then be derived expressing the state of inflation of the lungs at rest or at the end of forced expiration.

The method of bronchospirometry has also been explored in great detail. In general, the two techniques have been applied to different groups of patients. Bronchospirometry has found its greatest use in evaluation of unilateral or asymmetrical pulmonary or pleural disease in preparing and evaluating patients for thoracic operations. The residual volume has been examined most frequently for diagnosis of diffuse pulmonary disease.

The simultaneous and separate determination of the residual volume of each lung by bronchospirometry has received little attention. This neglect does not stem from any lack of important data which might be derived from this combination of procedures. Our own experiences have taught us that, with the use of conventional instruments and methods, two simultaneous residual volume studies through two lumens of a bronchospirometric catheter are time consuming and present many difficulties. Reliable methods for measuring the residual volume require considerable apparatus and careful gas analysis, while bronchospirometry involves tracheal intubation and the use of double recording devices. For determination of differential residual volume, a duplication of all apparatus and measurements becomes necessary.

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A recent review of the literature has revealed only two references to the determination of residual volume of the two lungs separately.[1] Björkman used a closed-circuit hydrogen dilution technique during bronchospirometry in six patients.[2] The method was applied to both lungs on two occasions. Further studies were not reported. More recently, Frenckner summarized results of differential residual volumes and total capacities of miners with respiratory complaints.[3] The method was not described, but it appeared to have been a closed circuit rebreathing technique like Björkman's. A Carlens catheter was used for separating the airways. The results suggested that, in spite of diffuse pulmonary disease, the total capacity of the two lungs may often differ significantly.

Examination of all the volume components of the two lungs separately became of interest at our laboratories during an evaluation of the effect of unilateral phrenic interruption. Electric stimulation of one phrenic nerve during bronchospirometry in man suggested that the decrease of total lung capacity following interruption may be due, in part, to impairment of mechanical function of the opposite side.[4] This impression was confirmed by observations on two patients in whom residual volumes and resting and exercise ventilations were determined separately for both lungs before and after phrenic interruption. Similar subsequent studies were fraught with difficulties. The catheter often changed position during the procedure, and the residual volume determinations by the closed-circuit oxygen rebreathing technique were unsatisfactory because proof of adequate gas mixture was not obtained.

More recently, we became interested in physiologic changes occurring in the remaining lung after pneumonectomy.[5] It appeared important to know the volume of all compartments of the remaining lung *before* the diseased lung had been removed. Postoperative studies alone have led to the conclusion that overdistention or emphysema had been caused by removal of the opposite lung. In reality this condition might have existed even before contralateral surgery.

Early difficulties in technique led to the establishment of four important steps for successful determination of the differential residual volumes:

(a) *Estimation of the extent of intrapulmonary mixing during or immediately after the procedure:* The residual volume cannot be measured directly by spirometry. Clinical methods for its indirect determination depend on dilution of the gas normally present in the lungs at the end of maximal expiration. With the closed-circuit method, the patient rebreathes into a spirometer containing oxygen until the nitrogen in the lungs comes into equilibrium in the lung-spirometer system.[6] The residual volume is calculated from the final nitrogen content and volume of the spirometer gas. Deceptively large volumes are obtained by this method when mixing of gases within the lungs is incomplete.[7] During our early experiments ventilation on the diseased side was often less than one liter per minute. Under these conditions there was presumably insufficient mixing with the spirometer gas on that side. However, the closed-circuit technique did not provide a measure of the effectiveness of this mixing.

(b) *Determination of the place in the respiratory cycle where the airway is switched to the residual volume apparatus:* This timing has a decided effect

on the finally determined volume. If the end of a quiet expiration is estimated by observation of the chest motion, faulty timing causes the largest single error in determining the functional residual capacity.[5, 8] This "switching error" becomes more significant as the functional residual capacity decreases and as the tidal volume increases. During bronchospirometry hyperventilation is usually marked and the functional residual capacity of each lung is small. The knowledge of the place in the respiratory cycle where the determination was begun is therefore important for later correction of the final volumes.

(c) *Obtaining of a bronchospirogram before the residual volume determination:* The appearance of the bronchospirogram is often the best evidence of correct placement of the catheter and of absence of leaks and technical difficulties.[9] When the residual volume was determined before conventional bronchospirometry the subsequent tracing often showed belatedly that technical errors had caused improper separation of the airways during the earlier residual volume determinations. Conventional spirometry, since it requires use of oxygen tensions larger than that in room air, cannot be used prior to residual volume determination because the lungs would be "contaminated" by gas of unusual and unknown composition. The calculations for the residual volume, determined by the open-circuit method, are based on the fact that the lungs normally contain 81 per cent nitrogen.

(d) *Automatic maintenance of proper catheter position within the tracheobronchial tree:* Once the residual volume determination has begun, interruption for repositioning of the catheter is not possible.

These requirements resulted in the development of techniques for separate and simultaneous determination of the functional residual capacities and for separate determination of intrapulmonary mixing indices of the two lungs which are the subject of this report.

MATERIALS AND METHODS

Functional residual capacity determination: The open-circuit method suggested by Darling, Cournand, and Richards[10] is used because it appears to be the most accurate clinical method and satisfies the first of the four criteria enumerated above, since it contains a provision for checking the adequacy of intrapulmonary mixing. The nitrogen, normally present in the lungs, is washed out by inhalation of pure oxygen. The expired gas is collected for seven minutes in a Tissot spirometer. The total amount of nitrogen present in the lungs at the instant the patient is switched to oxygen breathing is equal to the total amount of nitrogen collected in the spirometer. A small amount of nitrogen derived from the tissues and the inhaled oxygen can be calculated and subtracted. The volume of gas in the lungs, at the time the determination is begun, can be derived from the volume of nitrogen washed out of the lungs and the fact that the gas in the lungs originally contained 81 per cent nitrogen. An alveolar sample is collected at the end of seven minutes of oxygen breathing. This sample, in normal subjects, contains not more than 2.5 per cent residual nitrogen.[10] Larger amounts suggest insufficient intrapulmonary mixing. At our laboratories 1.5 per cent nitrogen is regarded as the upper limit of normal.[8]

To increase its accuracy the Darling method was modified in three ways.[8] Instead of a Haldane-Priestly tube an alveolar trap is used for terminal alveolar air sampling.[11] The tip of a curved glass tube with internal diameter of 2 cm. is submerged in acidulated water (Fig. 1). Observation of the bubbles emerging from the outlet permit estimation of the end

of the maximal expiratory effort. This is particularly helpful during differential residual volume determination. Collection of "alveolar" samples is assured even if one lung has a very small expiratory reserve or in patients who are short of breath.

For collection of the expired gas a Douglas bag is used instead of a Tissot spirometer. This eliminates spirometer rinsing, a large dead space correction and errors introduced by changes in the dead space as the water from the spirometer evaporates and the position of the spirometer changes.

Finally, a system is used which permits room air breathing and simultaneous graphic recording of respiratory excursions.[12] This twin drum-balloon method was devised in order to satisfy the foregoing criteria. The lungs are not contaminated with oxygen, and the system can be used for bronchospirometry prior to differential residual volume determination. Also,

APPARATUS FOR DIFFERENTIAL RESIDUAL VOLUME DETERMINATION

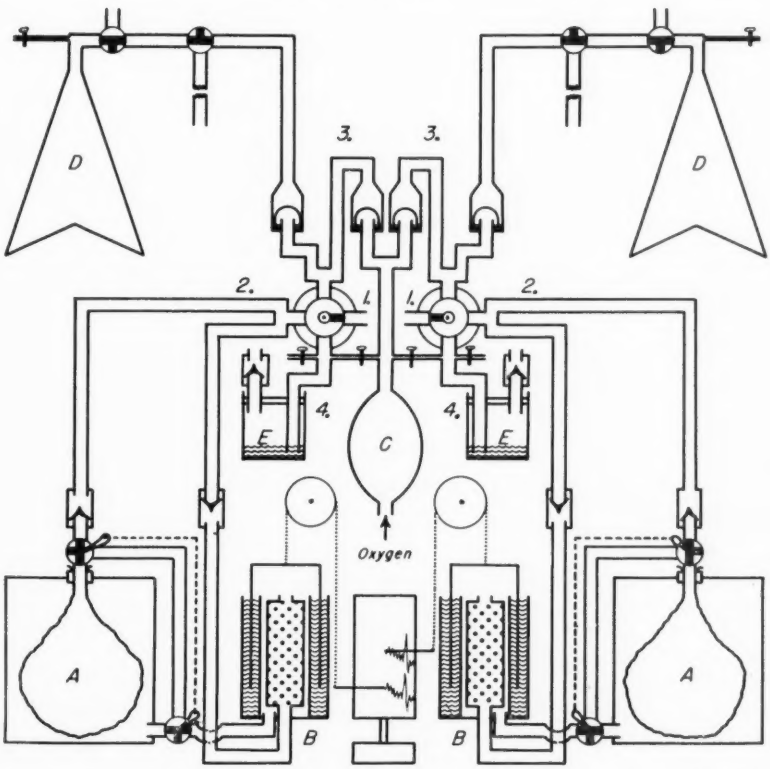

Fig. 1.—Diagram of apparatus for recording bronchospirometry and determination of differential residual volumes. Two five-way valves in the center provide a choice of four breathing circuits. The common terminals are attached to the two airways of the bronchospirometric catheter. Circuit No. 1 is used for room air breathing before and after the test. With the next circuit (No. 2) the patient breathes room air from the balloons (*A*) and exhales through the sodalime containers of the spirometers (*B*) into the boxes around the balloons. Recording of respiratory excursions is possible because the volume changes within the box are reflected by corresponding movements of the spirometer bells. With circuit No. 3 pure oxygen is inhaled from a common anesthesia bag (*C*) and the expired gas is collected in Douglas bags (*D*). The flow is directed by mercury-supported Bailey valves. On the last circuit (No. 4) the patient exhales forcibly through a water-sealed glass tube (*E*), a terminal alveolar sample is collected and the airway is returned to the initial circuit (No. 1). Conventional oxygen rebreathing bronchospirometry is possible by turning the four three-way valves which are coupled in tandem and are attached to the boxes.

the exact point in the respiratory cycle at which oxygen breathing was begun can be determined from the tracing. Correction of any error in judging the pulmonary midposition may be made.

A diagram of the entire apparatus is shown in Fig. 1. A portable instrument of this design can be rolled over the fluoroscopy table following introduction of the bronchospirometric catheter (Fig. 2). Two Douglas bags for collection of expired gas are suspended on either side. The anesthesia bag supplying pure oxygen to both lungs is in the background on the right. Two sets of mercury-supported Bailey valves direct the flow of oxygen. Two specially constructed five-way valves with large handles, seen in the center (Fig. 2), provide a choice of four breathing circuits (Fig. 1). Each has a common outlet for the bronchospirometric tube. In front of the valves are the two alveolar air traps dipping into jars containing acidulated water. The apparatus adds only 20 ml. of dead space to each lung. The bronchospirometric catheter diminishes the normal dead space by approximately an equal amount.

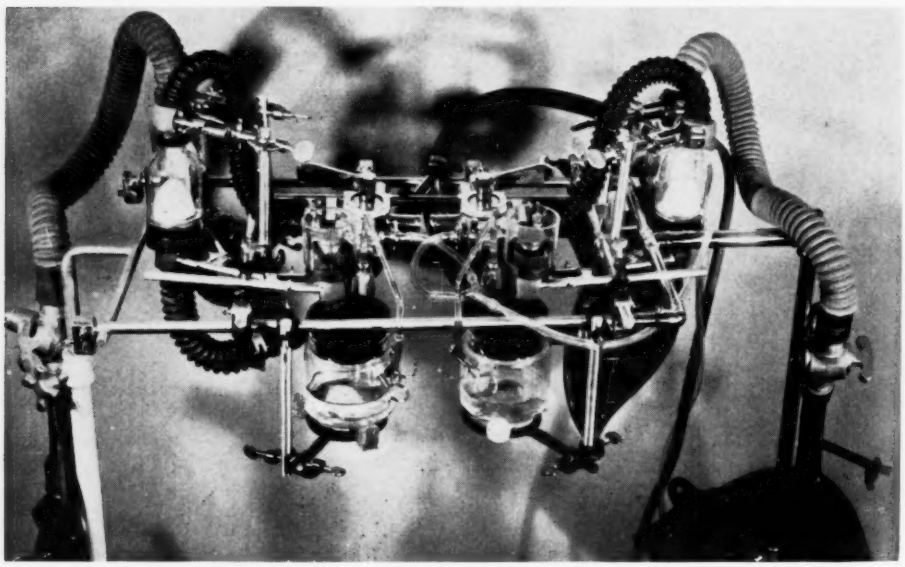

Fig. 2.—A portable apparatus which incorporates circuits Nos. 1, 3, and 4 shown in Fig. 1. The Douglas bags are suspended on either side. The anesthesia bag supplying pure oxygen is in the rear on the right. The five-way valves with large handles are in the center and the corresponding alveolar traps are supported directly in front of the valves. Two pairs of Bailey valves are seen immediately to the right and left.

The ambient air recording bronchospirometer: A twin spirometer incorporating two balloons suspended within airtight drums has been described.[12] The airway of each lung is connected to a balloon filled with room air. The patient inspires from the balloons and exhales through sodalime containers into the boxes around the balloons. The movements of spirometers connected to the boxes reflect the volume changes within the lungs. This apparatus is attached to the second circuit of the five-way valves (Fig. 1).

The double lumen catheter: The flexible bronchospirometric catheter of Carlens is well suited for this procedure.[13] Its rubber hook engages the carina as the tube is advanced and prevents passage of the catheter tip into the lower lobe bronchus. Gentle downward pressure during the procedure prevents the tip from slipping back into the trachea. The large internal diameters of the airways are important. With certain other catheters resistance breathing during bronchospirometry has been shown to cause a significant elevation of the functional residual capacity.[14]

The procedure: Prior to differential residual volume determination every patient is examined by conventional spirometry, and the combined residual volume of both lungs is determined in the conventional manner.

The large sized Carlens catheter is passed under topical anesthesia. The routine, outlined in a previous publication of this series, is followed except that Forrestier's solution is the preferred anesthetic.[9] The effect of dilute pontocaine may disappear before the entire examination has been completed. The patient then reclines on the fluoroscopy table. The lung volume apparatus (Fig. 2) is wheeled over the table and lowered until direct connection between the proximal orifices of the catheter and the common outlets of the five-way valves can be made. Slight downward pressure on the catheter is desirable. The twin spirometer is moved behind the table and the valved circuits from the balloons and to the boxes are connected (Fig. 1).

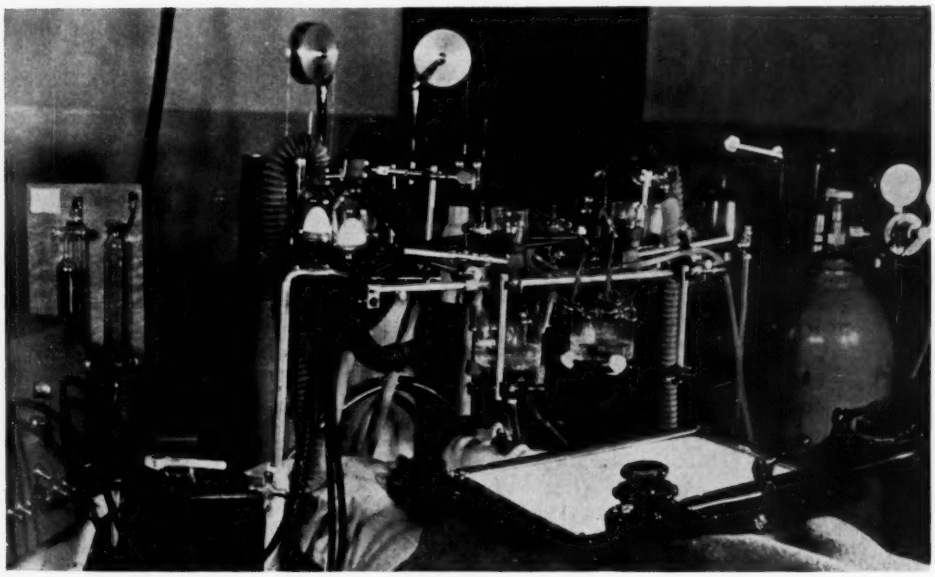

Fig. 3.—The entire apparatus is assembled and shown in use. The patient with the catheter in place rests under the fluoroscopic screen. The lung volume apparatus has been lowered over the patient's head. The twin spirometer is behind the table and boxes with balloons are mounted underneath the spirometer. The common oxygen supply is on the right and a double tonometer for collection of gas samples is in the rear on the left. In front on the left is a twin differential pneumotachograph for recording of instantaneous flow rates.

A patient reclining on the fluoroscopy table and all equipment is shown in Fig. 3. The patient's head with the catheter in place is seen behind the fluoroscopic screen in the foreground. The double lung volume apparatus is suspended over the catheter. The twin spirometer is behind the table. On the extreme right is the oxygen tank, the nitrogen content of which has been ascertained previously. A mercury-filled double tonometer for storage of alveolar or bag samples is shown in the background on the left. In front on the left is a twin differential pneumotachograph unit permitting recording of instantaneous flow rates during bronchospirometry.

Initially, the patient breathes room air from circuit No. 1 (Fig. 1). A spot film of the neck and upper chest is exposed.[9] The five-way valves are then turned simultaneously to circuit No. 2 and a bronchospirogram is recorded for four to eight minutes until an adequate base line has been obtained for determination of differential oxygen uptake. Ventilography is summate ventilation.[12] The interval is used to flush circuits No. 3 with oxygen.[8] Several vital capacity efforts are recorded at the end of this period. The five-way valves

are then turned to circuit No. 3, preferably at the end of a quiet expiration. The finally-determined volumes then correspond to functional residual capacities. This is not critical, however, since any "switching error" can be read from the graphs and the calculated volumes corrected accordingly. As the valves are turned to position No. 3 a timer is started and the oxygen flow is adjusted to maintain adequate filling of the anesthesia bag. During this period the alveolar traps (*E*) are flushed with oxygen and the patient is instructed concerning delivery of an alveolar sample. At the end of seven minutes the two valves are switched to circuit No. 4, and at the same time the patient performs a forced expiration. When gas bubbles cease to emerge from the glass tube (*E*) the two valves are quickly returned to the first, or room air circuit (Fig. 1).

If the initial bronchospirogram was not entirely satisfactory, or if an oxygen-breathing bronchospirogram is desired,[12] the valves are turned once more to the second (spirometer) circuit and another tracing is obtained. The four three-way valves, coupled in pairs, which link the boxes (*A*) to the spirometers (*B*) may be turned so that conventional rebreathing from the oxygen-filled bronchospirometer takes place (Fig. 1).

At the end of the procedure the Douglas bags (*D*) are removed for determination of the gas volume, and nitrogen content and samples are taken from the alveolar traps (*E*) for later analysis. Details of the sampling technique and analysis, of preparation of equipment and corrections are carried out as for combined lung volume determination.[8]

Difficulties: Adequate topical anesthesia is the key to an uneventful procedure. If the initial recording is disturbed by cough or complicated by accumulation of secretions the procedure must be discontinued. Air enters the system during cough even when the catheter balloons are adequately inflated. Avoidance of leaks is the principal problem, even in the absence of cough. This may be guarded against by repeated checking of cuff inflation and by preventing movement of the patient's head, the catheter, or the attached apparatus during the procedure.

In the absence of leaks the procedure is reliable unless one lung ventilates insufficiently to be flushed adequately in seven minutes. This is determined by results of analyses of the terminal alveolar samples. If both lungs are adequately ventilated neither sample contains more than 1.5 per cent nitrogen. If there are no leaks and both samples contain more nitrogen it can be assumed that a diffuse defect of intrapulmonary mixing exists; under these circumstances the mixing index after conventional two-lung residual volume determination should also have been high; the usual correction can then be made by subtracting the terminal alveolar nitrogen content from the initial 81 per cent.[10] If the sample from one lung is normal and the nitrogen content of the other is slightly larger, a unilateral intrapulmonary mixing defect exists and the usual correction can be made. However, if the alveolar sample from the diseased lung contains more than six or eight per cent nitrogen, adequate flushing on that side has not taken place and the results must be discarded. By comparing the lung capacities of the other lung with those obtained during combined residual volume determination it is possible to draw certain conclusions concerning the functional residual capacity of the defective lung.

Calculations: The calculations for each lung are the same as those outlined by Darling and associates for both lungs.[10] No corrections need be made for spirometer dead space since oxygen-flushed bags are used; final volumes are corrected for the "switching error." The total amount of nitrogen eliminated from the tissues during seven minutes is calculated according to empirical formula[10]; the amount excreted through each lung is apportioned according to the percentage of the total oxygen uptake of each lung during bronchospirometry since, in the absence of hypoxia, pulmonary blood flow on each side is proportional to the share of oxygen uptake.[1]

Treatment of data: All measured and derived data obtained from a typical normal volunteer are shown in Table I. The vital capacities and subdivisions are measured from the bronchospirogram for each lung. The percentage of the total volume contributed by each lung is calculated. The same measurements and calculations are made for minute ventilation and oxygen uptake. The nitrogen content of the terminal alveolar samples, deter-

TABLE I. DIFFERENTIAL RESIDUAL VOLUMES AND INTRAPULMONARY MIXING INDICES. COMPLETE DATA FROM NORMAL SUBJECT

	BRONCHOSPIROMETRY					SPIROMETRY		
	MEASURED VOLUMES			CALCULATED PER CENT OF THE TOTAL*		MEASURED VOLUMES	CALCULATED VOLUMES	
DETERMINATION	(1) TOTAL	(2) RIGHT	(3) LEFT	(4) RIGHT	(5) LEFT	(6) TOTAL	(7) RIGHT	(8) LEFT
Respiratory rate	10	10	10	--	--	10	–	–
Tidal volume, c.c.	530	265	265	50	50	520	260	260
Inspiratory capacity, c.c.	3,410	1,710	1,700	50	50	3,690	1,845	1,845
Expiratory reserve, c.c.	1,180	600	580	51	49	1,000	510	490
Vital capacity, c.c.	4,600	2,310	2,290	51	49	4,690	2,355	2,335
Functional resid. vol., c.c.	*2,060*	*1,050*	*1,010*	51	49	1,960	1,000	960
Residual volume, c.c.	–	–	–	–	–	960	490	470
Total lung capacity, c.c.	–	–	–	–	–	5,650	2,845	2,805
R.V./T.L.C. × 100, per cent	–	–	–	–	–	17	17	17
Ventilation, c.c./min.	5,300	2,650	2,650	50	50	5,250	2,625	2,625
Oxygen uptake, c.c./min.	250	126	124	50	50	236	118	118
Vent. Equivalent, L/100 c.c.	2.1	2.1	2.1	–	–	2.2	2.2	2.2
Mixing Index, per cent N_2	–	1.16	1.46	–	–	1.56	–	–

*The percentages of columns (4) and (5), derived from bronchospirometry, are applied the volumes obtained during spirometry (6) to obtain the final result of columns (7) and (8).

mined by gas analysis, was less than 1.5 per cent on both sides. The functional residual capacities for the two lungs are calculated from the corrected volumes and nitrogen content of the two Douglas bags. The calculated functional residual capacities are added and the contribution of each lung as per cent of the total is recorded (Table I).

The volume divisions of both lungs together were previously determined under basal conditions by combined spirometry and combined residual volume determinations (Table I). To determine the actual volume compartments of each lung *the percentages determined by split function studies are applied to these total volumes.* This is necessary because nonbasal conditions and resistance breathing invalidate actual volumes determined during bronchospirometry.[13, 14] The last two columns of Table I are derived in this manner, and these are the only data reported in subsequent tables.

RESULTS

We have measured differential residual volumes in various ways for more than four years. Only the recent results obtained by the present technique will be reported. The procedure was used on 40 occasions. In six patients the terminal alveolar sample on the diseased side contained more than 8 per cent nitrogen. Eight per cent was chosen arbitrarily as the largest acceptable value indicating adequate mixing. If the initial nitrogen concentration within the lung was not diluted at least ten times, we felt that insufficient flushing had taken place for reliable calculation of the functional residual capacities. The 34 acceptable studies involved 25 patients, six of whom were examined on two occasions and one was tested four times.

Normal distribution of the residual volume, functional residual capacity, and total lung capacity: The results of studies of four normal subjects are shown in Table II. There was considerable variation of the volume distribution between the two lungs among the four individuals. However, in each individual the various subdivisions of the lung volume were distributed almost identically. Consequently, the percentage of the total lung capacity occupied by the residual

TABLE II. DIFFERENTIAL RESIDUAL VOLUMES AND PULMONARY MIXING INDICES IN NORMAL SUBJECTS

PT. NO.	SEX	VITAL CAPACITY				RESIDUAL VOLUME				TOTAL LUNG CAPACITY				RESIDUAL VOLUME / TOTAL LUNG CAPACITY × 100			ALVEOLAR MIXING INDEX		
		RIGHT SIDE		LEFT SIDE		RIGHT SIDE		LEFT SIDE		RIGHT SIDE		LEFT SIDE							
		C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	RIGHT SIDE (PER CENT)	LEFT SIDE (PER CENT)	COMBINED SPIROMETRY (PER CENT)	RIGHT SIDE PER CENT N_2	LEFT SIDE PER CENT N_2	COMBINED SPIROMETRY PER CENT N_2
1.	M	2355	50	2335	50	490	51	470	49	2845	50	2805	50	17	17	17	1.16	1.46	1.56
2.	M	2320	60	1580	40	680	55	550	45	3000	59	2130	41	23	26	24	1.46	1.66	1.62
3.	M	2390	53	2070	47	610	54	530	46	3000	54	2600	46	20	20	20	0.96	0.82	1.10
4.	M	2070	54	1770	46	490	54	410	46	2560	54	2180	46	10	19	19	1.18	1.26	1.34
Mean		2284	54	1939	46	568	54	490	46	2851	54	2429	46	20	21	20	1.19	1.30	1.41

TABLE III. DIFFERENTIAL RESIDUAL VOLUMES AND MIXING INDICES IN UNILATERAL MODERATELY ADVANCED TUBERCULOSIS

PT. NO.	SEX	INV. SIDE	VITAL CAPACITY				RESIDUAL VOLUME				TOTAL LUNG CAPACITY				RESIDUAL VOLUME / TOTAL LUNG CAPACITY × 100			ALVEOLAR MIXING INDEX		
			INVOLVED SIDE		UNINVOLVED SIDE		INVOLVED SIDE		UNINVOLVED SIDE		INVOLVED SIDE		UNINVOLVED SIDE							
			C.C	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAI	INVOLVED SIDE (PER CENT)	UNINVOLVED SIDE (PER CENT)	COMBINED SPIROMETRY (PER CENT)	INVOLVED SIDE PER CENT N_2	UNINVOLVED SIDE PER CENT N_2	COMBINED SPIROMETRY PER CENT N_2
5.	F	L	1060	42	1470	58	500	50	500	50	1710	43	2270	57	29	22	25	1.92	1.20	1.41
6.	M	L	1590	54	1360	46	680	50	680	50	1880	52	1740	48	36	39	38	2.16	2.36	2.19
7.	M	L	1630	45	1920	55	420	44	530	56	2050	46	2450	54	21	21	21	1.52	1.42	1.76
8.	F	L	510	24	1610	76	305	37	515	63	815	28	2125	72	38	24	28	2.08	0.98	1.42
9.	F	R	860	44	1110	56	205	38	340	62	1270	44	1610	56	16	21	19	1.17	1.29	1.30
10.	F	R	1130	44	1430	56	410	54	360	46	1540	46	1780	54	27	20	23	0.97	0.94	1.14
11.	M	L	1370	38	2230	62	530	47	610	53	1900	40	2840	60	28	21	24	1.80	1.12	1.56
12.	F	L	1230	44	1570	56	510	52	470	48	1740	46	2040	54	29	23	26	1.26	0.96	1.36
Mean			1173	43	1587	57	445	47	510	53	1613	43	2107	57	28	24	26	1.61	1.28	1.52

volume (RV/TLC × 100) was almost identical on both sides in each case. All intrapulmonary mixing indices were well below the upper limit of normal. Although the nitrogen content of the terminal alveolar sample cannot be expected to be identical on the two sides, the highest two values and the lowest two values were found in the same two patients. Comparison of the mixing indices during bronchospirometry with that determined after the combined procedure showed that there was always more residual nitrogen after breathing oxygen with an ordinary mouthpiece than with a bronchospirometric catheter. This small but consistent different, both in normal volunteers and in many patients with pulmonary disease, was thought to be due to greater hyperventilation during bronchospirometry.

Localized unilateral parenchymal tuberculosis: Parenchymal disease does not affect lung function measurably until the process is well advanced.[1] In the eight patients whose studies are shown in Table III, the disease, judged by the roentgenograms, ranged from a minimal infiltration to involvement of not more than one-half of one lung field. The mean vital capacity on the affected side was slightly reduced in every case but one. The residual volume was compromised to an even lesser degree. It averaged 47 per cent of the total and in one-half of the patients it was as large or larger than that on the well side. Parenchymal disease apparently has a slight limiting effect on the inspiratory capacity. In some patients, because of bronchial involvement, retraction of the involved lobe, or impairment of pulmonary elasticity, the residual volume was actually larger on the involved side and suggested emphysematous changes. The fraction of total lung capacity taken up by residual volume was increased on both sides but more so on the involved side. The intrapulmonary mixing indices were within normal limits. The percentage of nitrogen retained in the lungs was somewhat larger on the affected side. A definite unilateral impairment of gaseous distribution was suggested in only two patients. The percentage of nitrogen obtained following breathing through a common airway compared well with the two indices during bronchospirometry.

Extensive unilateral parenchymal tuberculosis: The eight patients shown in Table IV had unilateral pulmonary disease involving most of the lung. Pleural disease, undoubtedly present in many, was thought to be incidental and was not responsible for the marked loss of function. All subdivisions of the total lung capacity were greatly impaired. The diseased lungs showed a mean vital capacity of only 367 ml., or 19 per cent of the total. Again, the residual volume was less reduced than the vital capacity, and the decrease of total lung capacity ranged between these two extremes. Combined study of both lungs showed that the residual volumes occupied more than its normal share of the total lung capacity. Differential studies revealed this disproportion to be more marked on the involved side. This was not due to an absolute increase of the residual volume but rather to a decrease of the total lung capacity and inability to expire a normal expiratory reserve volume. Differential alveolar mixing studies were most important in this group. After the usual combined determination only three patients showed more than 2.5 per cent nitrogen—the upper limit of normal.[10] In every one of these patients differential determinations

TABLE IV. DIFFERENTIAL RESIDUAL VOLUMES AND MIXING INDICES IN FAR-ADVANCED UNILATERAL DISEASE

PT. NO.	SEX	INV. SIDE	VITAL CAPACITY				RESIDUAL VOLUME				TOTAL LUNG CAPACITY				RESIDUAL VOLUME / TOTAL LUNG CAPACITY × 100			ALVEOLAR MIXING INDEX		
			INVOLVED SIDE		UNINVOLVED SIDE		INVOLVED SIDE		UNINVOLVED SIDE		INVOLVED SIDE		UNINVOLVED SIDE							
			C.C	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	INVOLVED SIDE (PER CENT)	UNINVOLVED SIDE (PER CENT)	COMBINED SPIROMETRY (PER CENT)	INVOLVED SIDE PER CENT N_2	UNINVOLVED SIDE PER CENT N_2	COMBINED SPIROMETRY PER CENT N_2
13.	M	R	560	25	1690	75	705	43	925	57	1265	33	2615	67	56	35	42	6.71	1.17	3.63
14.	F	R	220	13	1480	87	200	34	395	66	420	18	1875	82	48	21	26	5.71	1.06	2.12
15.	M	R	220	10	1980	90	205	13	1365	87	425	11	3345	89	48	41	42	-	-	-
16.	F	L	635	24	2005	76	245	35	505	65	880	26	2510	74	28	20	22	2.26	1.07	1.84
17.	F	L	155	14	955	86	70	12	500	88	225	11	1455	89	43	34	34	4.30	1.53	3.72
18.	F	L	550	25	1380	75	150	16	680	84	700	25	2060	75	21	33	30	1.45	1.30	1.88
19.	F	L	205	24	1605	76	45	8	495	92	250	12	2100	12	18	24	23	1.56	1.42	1.67
20.	F	L	390	23	1310	77	170	31	370	69	560	25	1680	75	30	22	24	2.90	1.70	2.68
Mean			367	19	1551	81	224	25	65	75	590	21	2205	79	37	29	30	3.56	1.32	2.51

revealed this finding to be solely due to unilateral defective gaseous distribution. Of even greater interest were those patients in whom the mixing index after the combined determination was normal, but in whom a distributional defect was demonstrated on the involved side by split function studies.

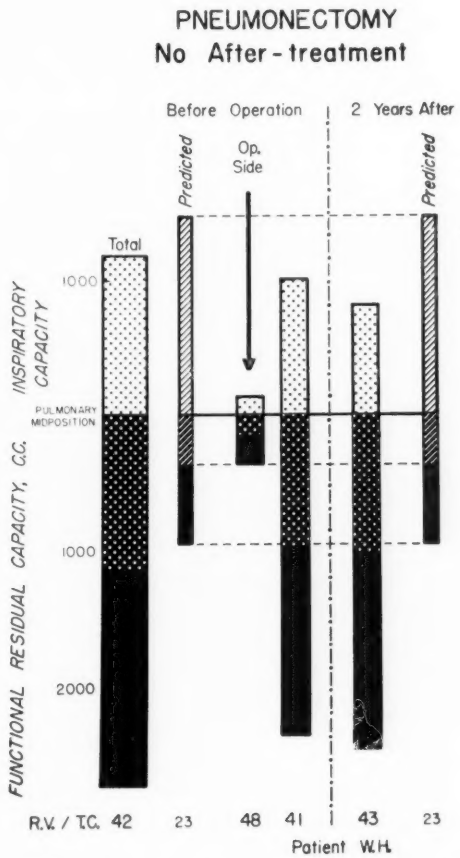

Fig. 4.—Differential lung volume studies before pneumonectomy showed a marked increase of residual volume and functional residual capacity affecting both lungs to nearly the same extent. Marked distention in the pulmonary resting position persisted in the remaining lung two years after operation.

The detailed findings in two patients with extensive unilateral pulmonary involvement prior to pneumonectomy are shown in Figs. 4 and 5. Both had studies of combined and differential residual volumes and bronchospirometry before operation and conventional volume studies at intervals after pneumonectomy.

The first (Fig. 4) showed a marked elevation of the functional residual capacity. The residual volume occupied 42 per cent of the total lung capacity. Split function studies before operation demonstrated that this hyperinflation of the lungs at the resting position was not due to obstructive emphysema on the involved side but rather to a bilateral process which involved the well as

TABLE V. DIFFERENTIAL RESIDUAL VOLUMES AND MIXING INDICES IN PATIENTS WITH PYOPNEUMOTHORAX

PT. NO.	SEX	INV. SIDE	VITAL CAPACITY				RESIDUAL VOLUME				TOTAL LUNG CAPACITY				RESIDUAL VOLUME / TOTAL LUNG CAPACITY × 100			ALVEOLAR MIXING INDEX		
			INVOLVED SIDE		UNINVOLVED SIDE		INVOLVED SIDE		UNINVOLVED SIDE		INVOLVED SIDE		UNINVOLVED SIDE							
			C.C.	PER CENT OF TOTAL	C.C	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	C.C.	PER CENT OF TOTAL	INVOLVED SIDE (PER CENT)	UNINVOLVED SIDE (PER CENT)	COMBINED SPIROMETER (PER CENT)	INVOLVED SIDE PER CENT N_2	UNINVOLVED SIDE PER CENT N_2	COMBINED SPIROMETER PER CENT N_2
21.	M	R	280	14	1870	86	280	29	680	71	560	22	2250	78	50	27	38	6.38	1.52	1.96
22.	F	R	145	11	1195	89	70	11	560	89	215	11	1755	89	33	32	32	2.89	1.51	–
23.	M	L	320	15	1820	85	350	35	650	65	670	21	2470	79	52	26	32	3.92	1.86	1.96
24.	M	R	230	18	1590	82	290	45	350	55	520	21	1940	79	56	18	26	4.39	0.96	2.04
25.	M	L	220	9	2190	91	390	42	550	58	610	18	2740	82	64	20	28	7.23	1.51	1.92
Mean			239	12	1733	88	276	33	558	67	515	19	2231	81	51	25	31	4.96	1.47	1.97

much as the diseased lung. A study of the remaining lung two years after pneumonectomy showed marked emphysema. The patient still breathed with a greatly inflated chest and the residual volume occupied 43 per cent of the total lung capacity. If differential lung volume studies had not been available prior to removal of the diseased lung it might have been concluded erroneously that the emphysema was the result of overdistention following pneumonectomy.

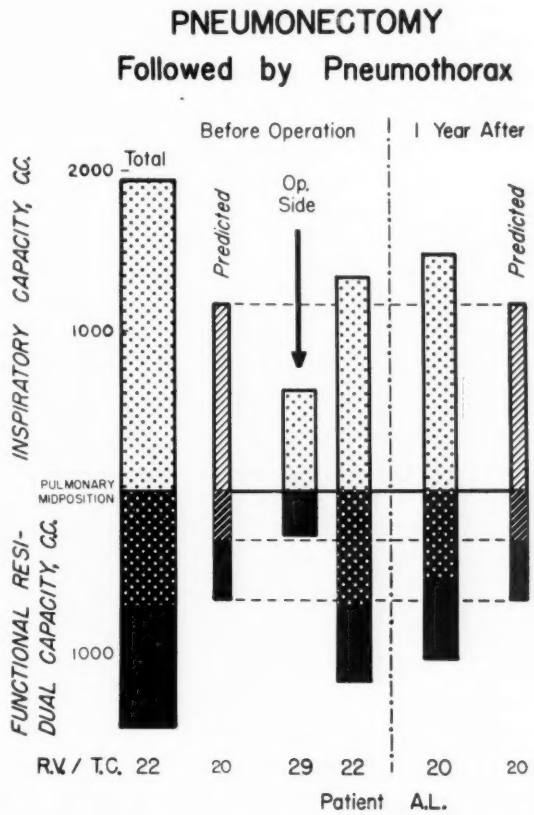

Fig. 5.—Normal distribution of the volume compartments of the unaffected lung before pneumonectomy was maintained for one year after operation by artificial pneumothorax on the side of pneumonectomy.

A second patient (Fig. 5) showed a normal distribution of the various volume compartments. The residual volume was increased relative to the total lung capacity on the diseased side only. After removal of this lung the space was treated by pneumothorax to avoid overdistention of the remaining lung. By comparing all the volume compartments of the remaining lung both before and after contralateral pneumonectomy it was possible to show that the total capacity was not altered one year after operation. Contralateral pneumothorax had not only prevented an increase of the residual volume and functional residual capacity but had brought about a small but definite favorable shift in this ratio.

Pleural involvement: Pleural disease has been shown to affect measurable function on the affected side to a greater degree than parenchymal disease.[1, 15] This was re-emphasized by the results of differential vital capacity studies of five patients with pyopneumothorax (Table V). The vital capacity averaged only 12 per cent, and was no more than 18 per cent of the total in any individual. In contrast to the previous group, the residual volume was only slightly affected. It averaged 33 per cent, and in individual patients was as much as 45 per cent of the total. This discrepancy was best reflected by the RV/TLC $\times$ 100 ratio. The mean residual volume occupied a normal 25 per cent of the total lung capacity on the unaffected side and 51 per cent on the diseased side. It is apparent that the volume of the "imprisoned" lung is not greatly decreased on fullest expiration and that the encroachment on the total lung capacity takes place almost entirely at the expense of the inspiratory capacity. The anticipated impairment of the mixture of gases under these conditions was confirmed by the alveolar samples obtained after seven minutes' oxygen breathing. Although normal on the good side, the mixing index was well above normal on the diseased side in every case. The mean alveolar nitrogen concentration was 4.96 per cent on the affected side and 1.47 per cent on the uninvolved side. Comparison of these values with the mixing index after combined study was of particular interest since the latter showed no deviation from the normal in any patient. Mixing defects which were unsuspected by the conventional determination were thus demonstrated by differential studies.

PREOPERATIVE AND POSTOPERATIVE STUDIES

The results of repeated studies before and after medical and surgical collapse procedures were of more than academic interest. It is generally accepted that a decrease in lung volume (collapse) promotes the healing of pulmonary tuberculosis.[16, 17] This decrease should be effective at rest and during limited activity which is the condition the patient with tuberculosis finds himself in most frequently. At the same time the collapse should not interfere with ventilation to the extent that moderate exertion incidental to daily activity becomes impossible. Finally, the collapse should be "selective" in the sense that the volume of the diseased portions of the lung should be reduced without causing unnecessary restriction of good portions of the same or of the opposite lung.

From these reflections a set of criteria can be evolved for changes in pulmonary volumina which would be of maximum benefit to the patient and, at the same time, cause minimal incapacity. Since the patient is at rest most of the day, the volume of the involved lung should be greatly reduced in the pulmonary resting position, i.e., the residual volume and the functional residual capacity on the involved side should be decreased. At the same time the ventilatory reserve should not be greatly impaired so that the patient will not be handicapped when sudden exertion or return to gainful employment becomes necessary, i.e., the inspiratory capacity should not be greatly decreased. Finally, the reduction of residual volume and functional residual capacity should be limited

to the involved side. Since all the volume compartments of both lungs can be measured separately, various collapse measures could be evaluated in these physiologic terms.

Phrenic interruption: Two patients were studied before and six weeks after interruption of the left phrenic nerve in an attempt to treat moderately advanced, unilateral pulmonary tuberculosis (Table VI). Before operation the first patient (No. 8) showed a marked diminution of vital capacity and residual volume on the left side while the second patient (No. 7) had an essentially

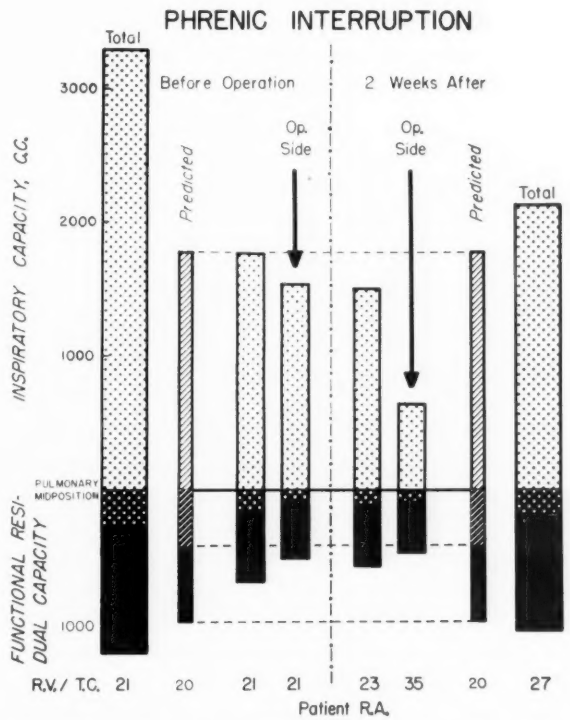

Fig. 6.—All volume compartments of both lungs separately are shown before and after interruption of the left phrenic nerve. The reduction of total lung capacity involved both lungs and took place at the expense of the inspiratory capacity. No "collapse" was obtained in the pulmonary resting position.

normal distribution of pulmonary volumina. After operation the vital capacity on the involved side was reduced to about one-half of its former value in each case, while the residual volume remained essentially unchanged. Because of the disproportion in reduction of these two volume compartments the RV/TLC ratio on the involved side rose from 38 to 50 per cent in one and from 21 to 35 per cent in the other patient.

These changes are appreciated more easily in terms of "collapse" if the total lung capacity and its subdivisions are presented in the form of block diagrams with the pulmonary resting position as a common base line. Spirometric and bronchospirometric findings of the second patient are shown (Fig. 6). The combined values for both lungs are represented by the wide block

TABLE VI. DIFFERENTIAL RESIDUAL VOLUMES AND INTRAPULMONARY

COLLAPSE PROCEDURE	SEX	SIDE	VITAL CAPACITY				RESIDUAL VOLUME			
			INVOLVED SIDE		UNINVOLVED SIDE		INVOLVED SIDE		UNINVOLVED SIDE	
			C.C.	PER CENT TOTAL	C.C.	PER CENT TOTAL	C.C.	PER CENT TOTAL	C.C.	PER CENT TOTAL
8. Before phrenic crush	F	L	510	24	1610	76	305	37	515	63
8b. After operation	F	L	270	15	1530	85	270	35	510	65
7. Before phrenic crush	M	L	1630	45	1920	55	420	44	530	56
7b. After operation	M	L	690	30	1600	70	410	47	470	53
5. Before extrapleural pneumothx.	F	L	1210	41	1770	59	500	50	500	50
5b. After operation	F	L	1060	37	1770	63	130	24	370	76
6. Before extrapleural pneumothx.	M	L	1590	54	1360	46	680	50	680	50
6b. After operation	M	L	1050	41	1530	59	330	41	480	59
10. Before intrapleural pneumothx.	F	R	1130	44	1430	56	410	54	350	46
10b. After injection	F	R	540	29	1310	71	270	44	350	56
11. Before thoracoplasty	M	L	1370	38	2230	62	530	47	610	53
11b. After 1st stage	M	L	860	32	1840	68	450	43	600	57
11c. After 2nd stage	M	L	580	23	1920	77	310	29	760	71
11d. Six months later	M	L	690	26	1960	74	440	45	540	55
12. Before thoracoplasty	F	L	1230	44	1570	56	510	52	470	48
12b. After 7 rib thpl.	F	L	580	29	1420	71	350	43	470	57

on the extreme left before operation and on the extreme right after operation. The total lung capacity has been reduced at the expense of the inspiratory capacity. The expiratory reserve volume and the residual volume have remained almost unchanged. Differential studies show that this over-all reduction was not due to a decrease on the affected side alone. The inspiratory capacity decreased 940 ml. on the side of phrenic interruption and 320 ml. on the opposite side. When these results are compared with the previously enumerated desirable changes it is apparent that the opposite of the ideal effect has been attained. The pulmonary midposition (functional residual capacity) has not been decreased and no collapse exists while the patient is at rest. The inspiratory capacity has been sharply reduced. The patient's ability to meet unusual ventilatory demands has been impaired. Worst of all, the reduction of maximal pulmonary expansion has not been limited to the affected side. The mechanism of the mechanical impairment on the opposite side was investigated on the basis of previous bronchospirometric studies. It is thought to be due to the swinging motion of the mediastinum following phrenic interruption.[4]

Extrapleural pneumothorax: The opposite type of change was observed after another more selective type of collapse: extrapleural pneumothorax. Two patients (No. 5 and No. 6) were studied before and after extensive extrapleural pneumonolysis (Table VI). The detailed results of the first case are shown diagrammatically (Fig. 7). The total lung capacity was reduced as much as by phrenic interruption, but the reduction took place almost entirely at the expense of the functional residual capacity and residual volume. The patient's ability to meet emergencies has not been impaired while in the usual resting

MIXING INDICES BEFORE AND AFTER COLLAPSE PROCEDURES

TOTAL LUNG CAPACITY				RESIDUAL VOLUME / TOTAL LUNG CAPACITY × 100			ALVEOLAR MIXING INDEX		
INVOLVED SIDE		UNINVOLVED SIDE							
C.C.	PER CENT TOTAL	C.C.	PER CENT TOTAL	INVOLVED SIDE (PER CENT)	UNINVOLVED SIDE (PER CENT)	COMBINED SPIROMETRY (PER CENT)	INVOLVED SIDE PER CENT N_2	UNINVOLVED SIDE PER CENT N_2	COMBINED SPIROMETRY PER CENT N_2
815	28	2125	72	38	24	28	2.08	0.98	1.42
540	21	2040	79	50	25	30	–	–	–
2050	46	2450	54	21	21	21	1.52	1.42	1.76
1100	35	2070	65	35	23	27	1.92	1.76	1.96
1710	43	2270	57	31	20	25	1.92	1.20	1.41
1190	34	2140	66	12	18	15	0.98	1.26	1.16
1880	53	1740	47	36	39	38	2.16	2.36	2.19
1380	41	2010	49	24	24	24	1.92	1.96	1.76
1540	48	1780	52	27	20	23	0.97	0.94	1.14
810	33	1660	67	33	21	25	0.90	0.80	1.42
1900	40	2840	60	28	21	24	1.80	1.12	1.56
1310	35	2440	65	34	25	28	2.67	1.45	1.67
890	25	2680	75	35	28	30	2.90	1.40	2.01
1130	32	2500	68	39	22	27	2.41	1.96	1.78
1740	46	2040	54	29	23	26	1.26	0.96	1.36
930	33	1890	67	38	25	29	2.83	1.46	1.29

condition the volume of the affected lung is less than one-half of its previous value. Because the "collapse" has taken place at the opposite end of the pulmonary volume scale the residual volume/total lung capacity ratio has not been increased, as after phrenic interruption, but has been reduced from 31 to 12 per cent in one case and from 36 to 24 per cent in the other. The procedure in a sense is also more "selective" since the changes have been limited to the operated side. These laboratory findings coincide with lung volume studies done on a patient where extrapleural pneumonolysis was performed on a single remaining lung.[18] The selectivity of the operation and its function-sparing characteristics are borne out by clinical experiences.[19, 20]

Intrapleural pneumothorax: The findings after introduction of air in the intrapleural space (Table VI) confirm the impressions of others that the effect of this procedure is not limited to the involved hemithorax.[1] Both the vital capacity and the residual volume are reduced on the treated side, but a disproportion, unfavorable to the vital capacity, results in a moderate elevation of the residual volume/total lung capacity ratio. The findings in this patient should be viewed with the knowledge that postoperative studies were carried out when only a narrow rim of unselective pneumothorax existed. A third study of this patient, when the lung was about 50 per cent collapsed, was not satisfactory because the lung did not ventilate enough to be adequately flushed during the seven-minute oxygen breathing period.

Staged thoracoplasty: Studies performed before, after every stage, and six months after operation (Table VI) suggest that destruction of a portion of the thoracic cage causes severe limitation of maximal expansion on that side.

This was repeatedly shown by staged bronchospirometric study of over 200 patients before and after thoracoplasty.[21] The residual volume participates in this collapse to a lesser extent and recovers more postoperatively. The RV/TLC ratio, somewhat increased shortly after operation, is further increased six months later. Another observation, not previously recorded, appears to be peculiar to immobilization of the hemithorax. Both patients (No. 11 and No. 12)

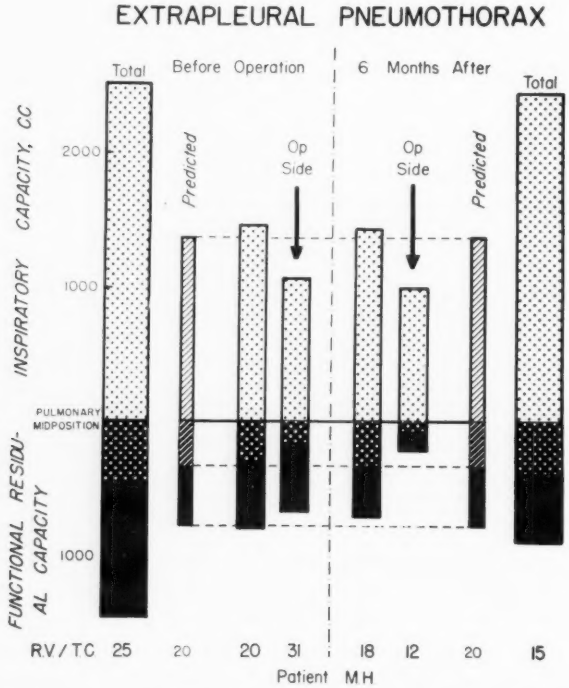

Fig. 7.—The reduction of total lung capacity after extrapleural pneumothorax was confined to the involved lung and took place chiefly at the expense of residual volume and expiratory reserve volume. Marked "collapse" was achieved in the pulmonary resting position without encroachment on the ventilatory reserve.

had normal intrapulmonary mixing indices before operation although the nitrogen percentage was slightly higher on the diseased side. Shortly after each stage, patient 11 showed not only an over-all increase of the index, but intrapulmonary mixing became definitely abnormal on the operated side. This was presumably due to paradoxical motion of the chest wall. However, the nitrogen content of the alveolar sample remained permanently elevated on that side although the over-all value returned to normal.

The frequently voiced belief that collapse due to thoracoplasty takes place chiefly at the expense of the residual volume appears to be mistaken. On the contrary, in view of the increased intrapulmonary mixing index and the elevated ratio of residual volume to total lung capacity, the operation should not be expected to correct pulmonary emphysema as is frequently claimed. This condition may be expected to be aggravated by thoracoplasty, an expectation borne out by clinical experience.[21]

That thoracoplasty may permanently diminish the vital capacity and residual volume of the opposite lung has been demonstrated by serial studies of patients with pneumonectomy before and after space-filling thoracoplasty.[5] The results outlined in Table VI suggest that this may hold true also if staged partial thoracoplasty is performed on the opposite side over diseased lung. The musculo-skeletal factors which may be responsible for these contralateral effects have been discussed.[5, 21, 22, 23]

SUMMARY

An open-circuit technique is described for the simultaneous and separate determination of the residual volume of each lung. The method includes provisions for estimation of the effectiveness of intrapulmonary mixing, for determination of the place in the respiratory cycle where the examination is begun, for recording bronchospirometry before lung volume study and for maintenance of proper catheter position.

In four normal volunteers the functional distribution between the two lungs differed considerably. In each individual, however, the percentage of the total inspiratory capacity, expiratory reserve volume and residual volume contributed by a single lung was the same so that the ratio of residual volume to total lung capacity was the same on both sides.

In eight patients with localized unilateral parenchymal tuberculosis the vital capacity was only slightly reduced and the residual volume was little affected or even increased. Intrapulmonary mixing was normal on both sides.

In eight patients with extensive unilateral parenchymal disease the vital capacity was severely reduced and the residual volume was moderately decreased. Intrapulmonary mixing was impaired occasionally on the involved side.

The disproportionate reduction of vital capacity and residual volume was most marked in five patients with "imprisoned" lungs due to pyopneumothorax. On the affected side the vital capacity was only slightly larger than the resting tidal volume while the residual volume often amounted to nearly one-half of the residual volume of both lungs together. Intrapulmonary mixing was greatly abnormal on the diseased side although conventional determination of this mixing index for both lungs together often failed to bring out this defect.

The effect of pneumonectomy on the remaining lung could be studied since it was possible to measure all volume compartments of this lung before the other lung had been removed. Overdistention and emphysema after operation at times existed to the same degree before removal of the other lung.

A number of collapse procedures used in the treatment of pulmonary tuberculosis were evaluated. Phrenic interruption caused a reduction of the inspiratory capacity which was not limited to the operated side. Extrapleural pneumothorax reduced the lung volume at the expense of the residual volume and expiratory reserve volume and the effect was confined to the operated side. Immobilization of a portion of the chest wall by thoracoplasty caused reduction of all volume compartments. Both the intrapulmonary mixing index and the ratio of residual volume to total lung capacity increased.

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ULTRASONIC VISUALIZATION OF SOFT TISSUE STRUCTURES OF THE BODY

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INTRODUCTION

THE great advances in the field of exact medical diagnosis which have been made possible through the general application of radiology, endoscopy, and other visualization techniques are well known, yet many deficiencies still exist. It is a too frequent occurrence for the physician to leave the autopsy room wishing that the facts revealed there could have been known by him before he started therapy on his patient. In an x-ray film of the leg, for example, a clear view of the fine details of the epiphysis, cortex of the diaphysis, and even the tiny trabeculae makes examination of the bone easy. In contrast, the ligaments, tendons, nerves, muscles, and other soft tissues surrounding the bone are barely visible, and their true structural details are never seen. This is because their densities are so similar to those of the contiguous tissues that there is essentially no difference between the amounts of roentgen rays absorbed in passage through these various structures. Although adjacent areas may differ in density by as much as 10 per cent, they often show insignificant difference on the x-ray. The need for visualization of detail is even more important in studies of such organs as the thyroid, spleen, liver, pancreas, breast, etc.

It is known that when a pulse of ultrasound passes through soft tissues echoes of sizeable amplitude are produced. These echoes can be seen on a cathode ray oscilloscope, and appear much like the waves of an electroencephalogram. The application of this ultrasonic phenomenon to diagnostic medicine has not been very successful because the complexity of the sound-reflecting structures produces echoes which are so numerous and variable that controlled experiments and consistent results are difficult to obtain. The value of such a system would be greatly enhanced if a true image similar to a roentgenograph or photograph could be obtained, for it would then be possible to visualize normal and pathologic soft tissue structures, nonmetallic foreign bodies, nonopaque gallstones, etc. We have developed such an instrument which we have named the Somascope (tissue vision). It is the purpose of this study to show that this instrument is capable of making soft tissue structures visible in a manner which is useful for diagnostic purposes.

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METHODS

Ultrasonics is the term applied to sound waves above the frequency which can be heard by the human ear; that is, above 18 to 20 thousand cycles per second. Ultrasonic waves at a frequency of a few million cycles per second exhibit interesting quasi-optical properties. At these frequencies they can be confined to a narrow beam by lenses and focusing reflectors. As with light waves, diffractive, refractive, and interference effects take place. At these high frequencies "sound" waves are unable to travel through gases, due to extreme absorption, but may be transmitted freely through liquids and solids. The use of these waves for medical diagnosis was indicated by the relative ease with which they are reflected by small objects differing in acoustic properties (principally density) from their surroundings by as little as one part in a thousand.

Fig. 1 shows how ultrasonic waves are utilized in the Somascope. A short pulse of electrical energy is produced and then converted into a very short burst of ultra high frequency sound in the head of the instrument. This ultrasonic pulse is concentrated in a very narrow path, or beam, by "sound" lenses, and directed through liquid into the object

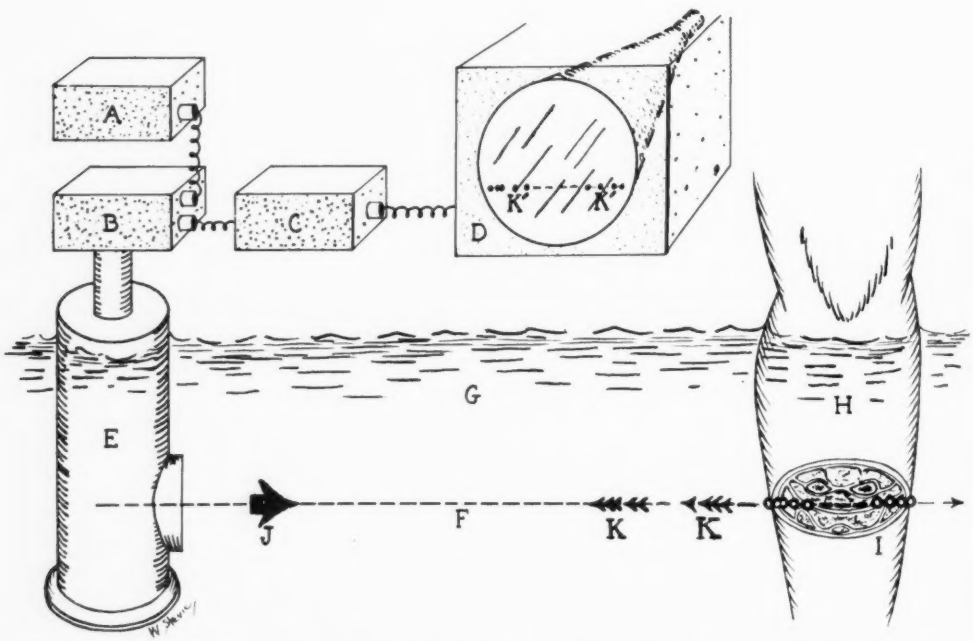


Fig. 1.—Block diagram of the first steps in the production of a cross-section somagram of the forearm. A very short and powerful electrical pulse is formed in the pulse generator (*A*). This electrical pulse is then passed into the "sound head" (*E*). In the sound head, the electrical pulse is converted into a short burst of ultra-high frequency sound (*J*) which is focused into a narrow beam so that it follows a linelike path (*F*). When the finite pulse strikes the skin surface of the forearm (*H*), the first of a series of echoes is formed and reflected back toward the sound head. The majority of the pulse beam penetrates the forearm. At each point within the arm, where the beam passes into a new structure which has sonic properties different from those in which the pulse wave was travelling, a new echo is formed; these points are shown in the diagram by small circles, and the echoes produced are represented by the small arrows (*k-k*). The Echoes produced return to the sound head (*E*), where these mechanical echo pulses are converted into their electrical equivalents which are then returned to an amplifier (*C*). After amplification, the electrical echo chain goes to the cathode ray oscilloscope (*D*), where it causes a series of bright spots (*k'-k'*) to appear on the face of the cathode ray tube. These spots (*k'-k'*) are identical to the sonic echoes (*k-k*) which were produced by the tissue boundaries in the forearm. The line of bright spots thus form one line of the cross-section picture to be produced on the tube face. The direction and distances between the spots correspond exactly to the direction of the path of the sound beam through the arm and the distances between the tissue structures.

to be examined. At the surface, and at every point within the object where the sound pulse goes into a structure having different acoustic properties, a portion of the energy is reflected back as a short, discrete echo. The echo then travels back to the head of the instrument where it is received and again converted into an electrical pulse. Additional echoes are produced at similar partially reflecting surfaces by the same outgoing sound pulse as it traverses consecutively deeper structures. These echoes likewise return to the head of the instrument and are converted into a train of electrical signals (pulses). This train of signals is amplified and is passed into a cathode ray tube where it produces a series of bright spots. The distance between these spots corresponds closely to the distance between the reflecting surfaces in the line taken by the sound pulses. The direction of the line of spots on the picture tube corresponds to the direction the beam took through the structure to be examined, and thus forms one line of a picture. As soon as all of the echoes have returned to the receiver, a second sonic pulse is formed and the entire process is repeated (Fig. 2). Thousands of pulses are generated in this same manner every second. Each of these pulses is caused to take a slightly different path through the tissue so that it passes in a line adjacent to the previous one. Thus the beam of pulses is swept or scanned

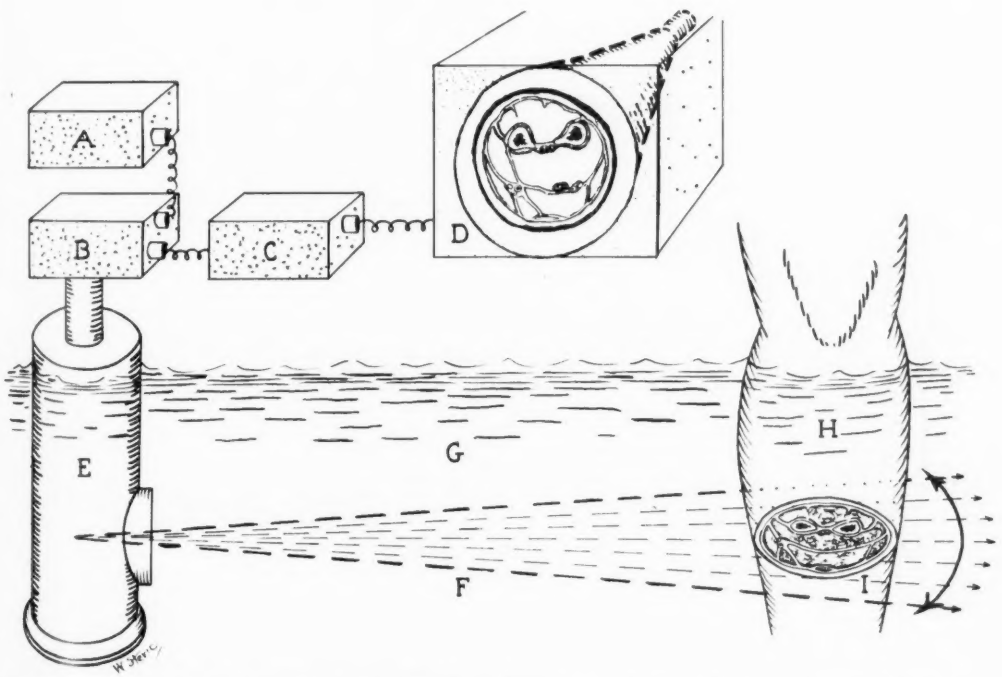


Fig. 2.—Block diagram of the method used in the production of a cross-sectional somagram of the forearm. As soon as the first line of bright spots, or picture elements, has been formed on the cathode ray tube (*D*), as shown in Fig. 1, a new electrical pulse is formed in the pulse generator (*A*). This pulse is again transformed into a very short ultrasonic pulse and focused into a beam by the submerged sound head (*E*). This new pulse, however, is forced to take a slightly different course (*F*) through the liquid bath (*G*) and arm (*H*) than that taken by the previous pulse. The echoes formed by the tissue boundaries in this new path are again received by the sound head (*E*) where they are transformed into a new chain of electrical signals which are amplified and shown as a new line of bright spots on the cathode ray tube (*D*), adjacent to the previous line, but corresponding to the path of the sound beam through the arm. The process of pulse formation is repeated thousands of times a second and each pulse takes a slightly different path (*F*) so that it passes in a line adjacent to the previous pulses as the beam is swept through the arm in a two-dimensional plane (*I*). The position of the beam during this sweeping is accurately copied by the lines of spots on the cathode ray "picture tube" (*D*). Due to the rapid rate of formation of these spots and lines on the picture tube, they can be made to appear as a visually continuous picture.

through the entire object. Altogether these pulses, by sonic probing, explore the entire object in a two-dimensional plane. The position of the beam during its scanning motion is accurately copied by the lines of spots on the picture tube. These spots produce a two-dimensional, cross-sectional picture of the sonically reflecting surfaces within the structure scanned by the sound beam. It is possible to repeat the entire process of picture formation many times a second, producing a visually continuous image on the picture tube, very much as is done in television.

In practice, a laboratory model is necessarily somewhat more complex than the simple explanation of the mechanism would suggest (Fig. 3). Many separate chassis and cabinets are used to house the various functional parts of the unit in order to permit modifications and repairs. Fifty-eight vacuum tubes are used to produce, control, amplify, synchronize,

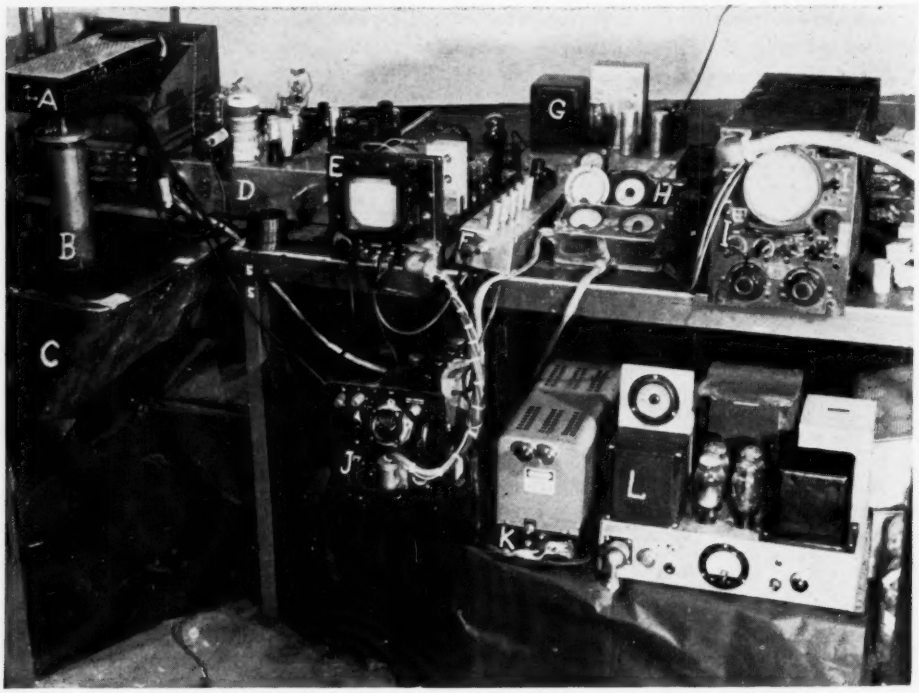
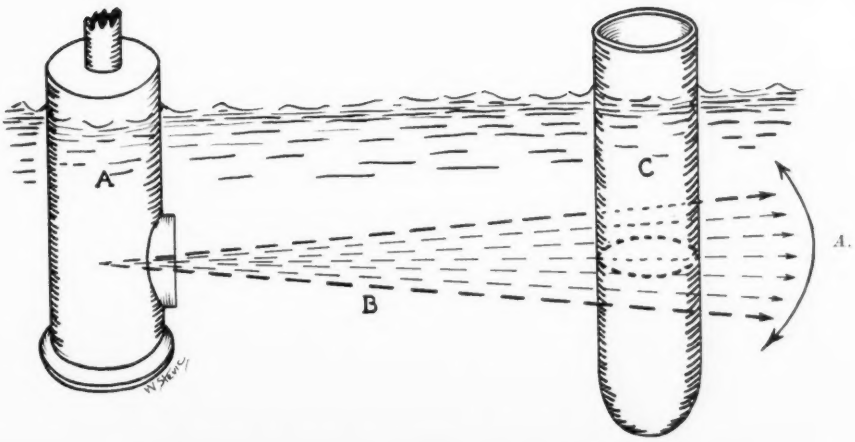
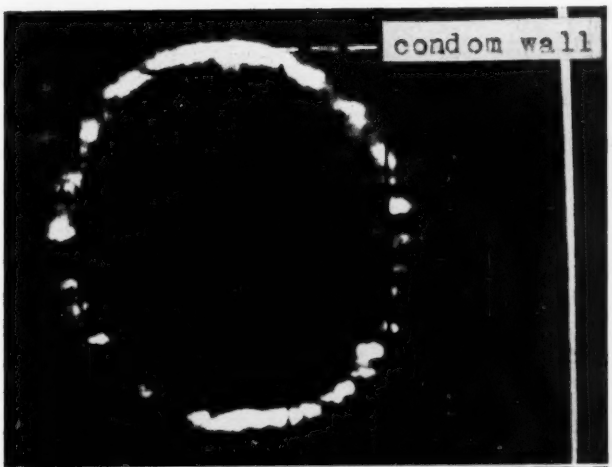


Fig. 3.—Laboratory equipment designed for the production of somagrams. *A*, Horizontal beam scanning and synchronization system; *B*, waterproof sound transducer, three-element focusing system, amplifier protection system, and pre-amplifier; *C*, tub for immersing tissue to be examined; *D*, repetition rate generator, high voltage pulse generator, oscilloscope synchronization, and delay system, etc.; *E*, cathode ray oscilloscope for viewing Somagrams; *F*, main amplifier; *G*, pre-amplifier power supply; *H*, meter control system; *I*, monitor oscilloscope; *J*, voltage regulated power supply for cathode ray oscilloscope; *K*, high-voltage power supply; *L*, main power supply.

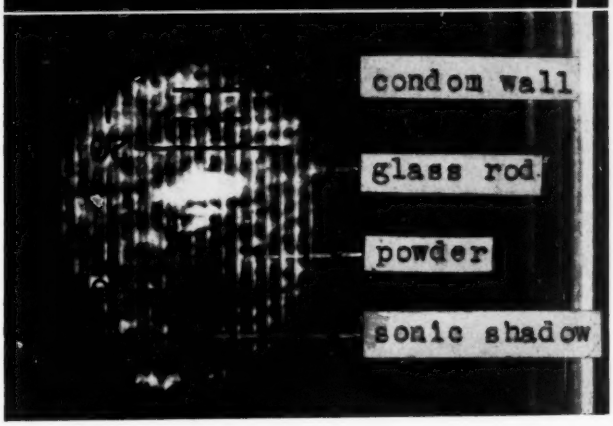
scan, and monitor the various electrical pulses in this unit. Time and financial limitations have caused some losses in sensitivity and stability in the laboratory model, but these are being corrected in a new model. It should be remembered that the ultrasonic pictures on the following pages represent some of the first pictures known to have been made by this method, and should therefore be compared to the early x-ray plates, rather than to the highly perfected ones produced by modern roentgen equipment. The first Somagrams were produced in 1950[29] and subsequent improvement has been very rapid. Operation of the home-made units and associated test instruments has given evidence of many ways in which further improvements can be obtained.



A.



B.



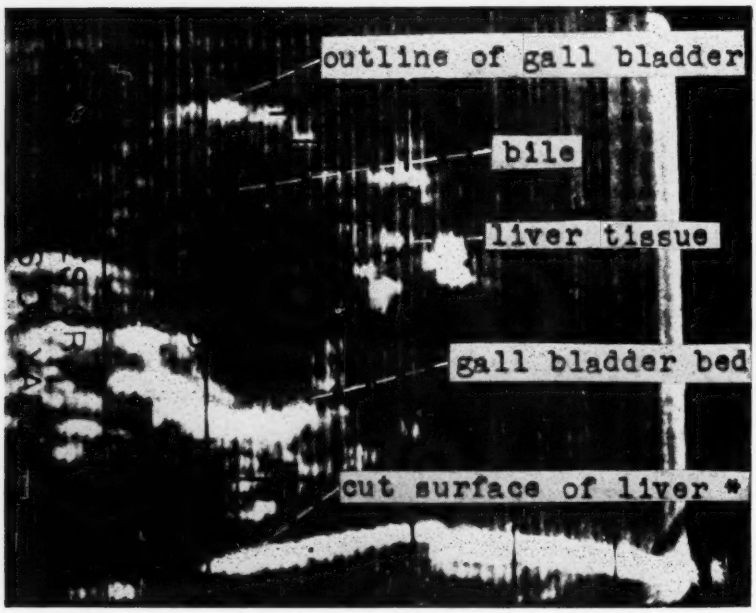
C.

Fig. 4.—Diagram of the method of scanning a simple test object and results obtained. *A*, The ultrasonic pulse generator is shown at (*A*). Each pulse produced travels in a narrow path and strikes the fluid-filled condom (*C*). Those pulses which strike the midportion of the condom produce one echo at the front surface where the beam penetrates the rubber membrane, and a second when it penetrates the far side. Each pulse, therefore, forms two spots on the cathode ray picture tube. The sweeping, or scanning, motion of the multiple beams is diagramed by the "dashed" lines (*B*). Combined, they explore a cross-section circle of the condom as shown by the "dotted" line. The Somagram which is produced on the scope tube should be a simple circle. *B*, Photograph of the cathode ray picture tube showing a cross section of a condom. *C*, Photograph of the cathode ray tube face while a condom was being scanned. A fine talcum powder has been mixed with the water in the condom and a glass stirring rod placed in the center. (The straight black lines and numerals which can be seen in portions of the somagrams are calibration markings on the synchroscope tube and should be considered as removable artifacts.)

RESULTS

Fig. 4, *A* shows a simple test object, a condom, being held in the scanning beam. It will be noted that the plane of intersection is such that the sound pulses pass through a circular cross-section. Fig. 4, *B* is a photograph of the picture tube displaying this circular cross-section of the condom.

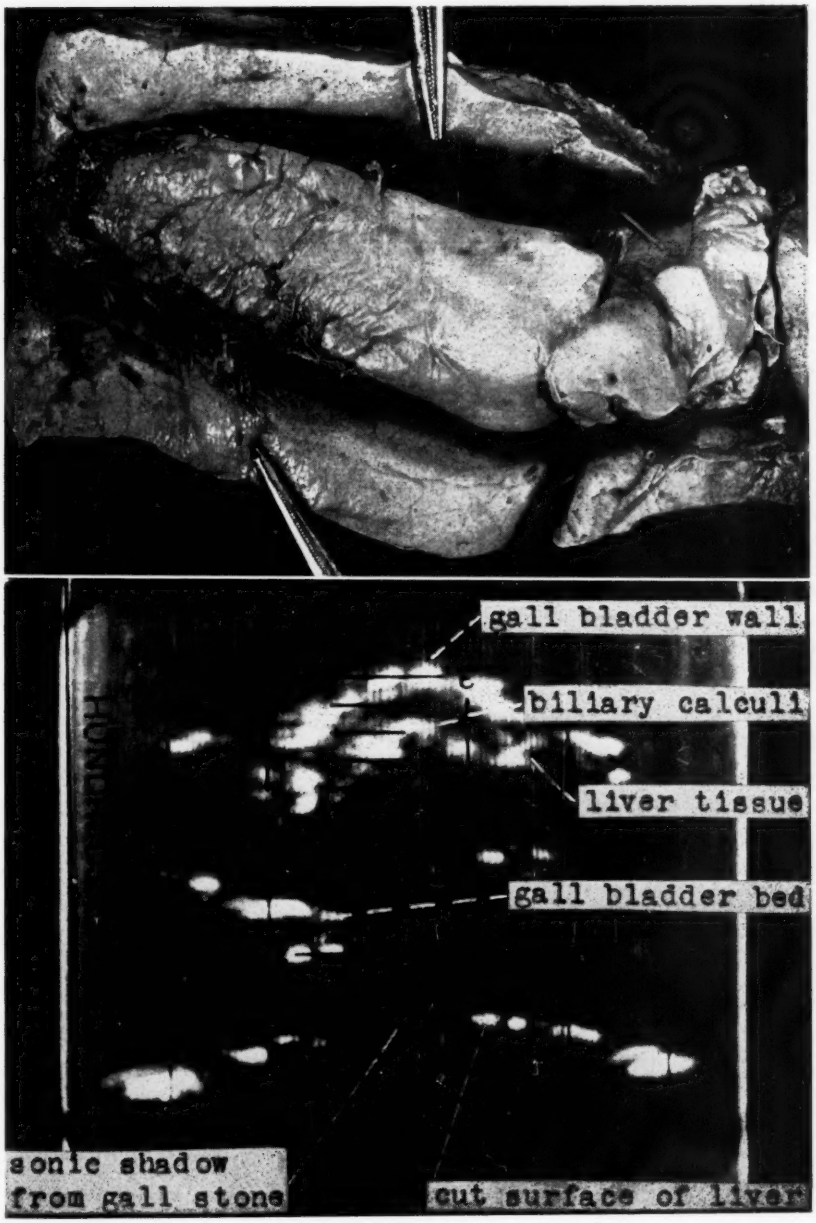
A.



B.

Fig. 5.—*A* and *B*, studies on a normal and pathologic gall bladder. *A*, A normal, bile-filled gall bladder with an attached block of liver. *B*, Cross-section Somagram. In cross section, the thin-walled gall bladder produces a circular outline much like the cross section of the condom.

C.



D.

Fig. 5.—*C* and *D*, studies on a normal and pathologic gall bladder. *C*, Thick fibrous-walled contracted gall bladder (attached to block of liver) which contains numerous large faceted and small gravel-like calculi. *D*, Cross-section Somagram. The thick, fibrous wall of the gall bladder is well seen. Inside the gall bladder, strong echoes from the large faceted stones and gravel appear. The broken line of the gall bladder bed and the cut surface of the liver is the result of the areas of sonic shadow produced by the highly reflecting calculi.

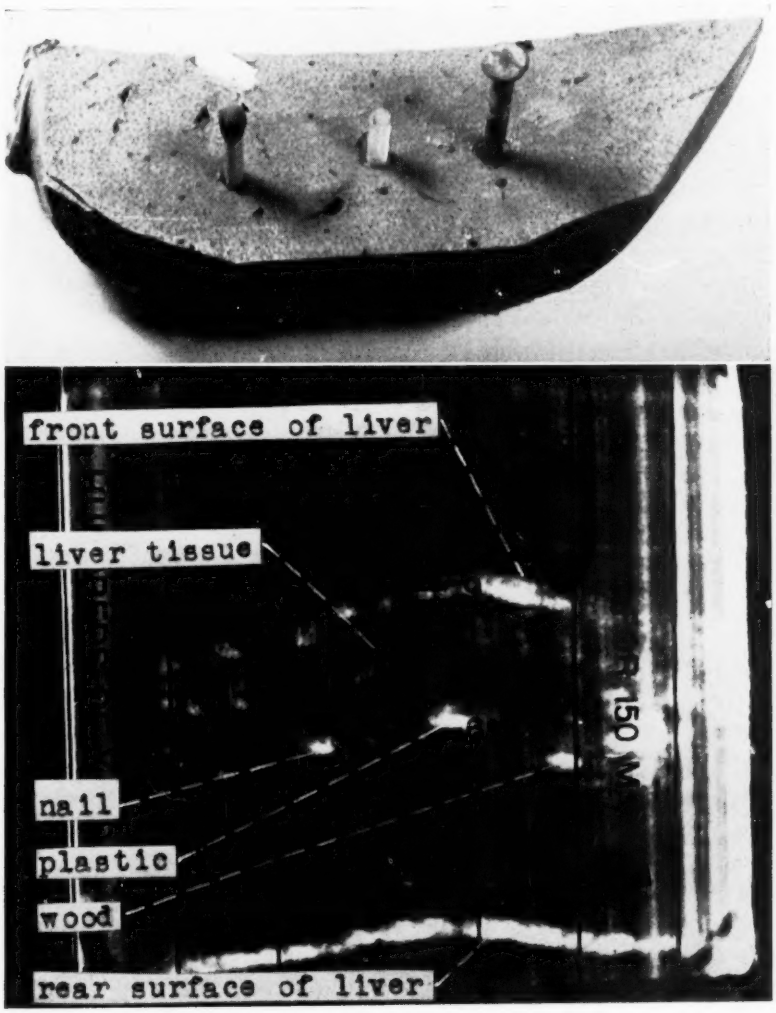
To produce the ultrasonic picture shown in Fig. 4, *C*, the condom is again held in a vertical position, a glass stirring rod is placed in its center, and a very fine powder (talc) is mixed with the water in the condom. The circular wall of the condom is again shown; a strong signal from the glass rod appears in the center of the circle, a fine, diffuse signal caused by the powder may be seen throughout the rest of the condom. It should be noted that nearly all of the sonic pulse is reflected by the glass rod; an area of "sonic shadow" is thus produced behind the rod. At higher powers this shadow effect is marked. The far wall of the condom is not seen in the ultrasonic picture where it lies in the shadow of the glass rod. This "shadowing" effect would also be seen in the picture if some small air-filled object, such as a soda straw, had been placed in the condom. This effect demonstrates one limitation of this method. In its present form it is not suited to visualization of the internal structure of bones or air-filled cavities. The shadow produced by sonically-opaque objects is an important aid in their identification, since it separates them from all forms of semi-fluid material. This will be shown in later illustrations.

A normal gall bladder is shown in Fig. 5, *A*. The thin wall of this structure would be expected to produce a narrow, continuous, circular line much like that produced by the condom shown in Fig. 4, *B*. This is verified in Fig. 5, *B*. In this Somagram, there is a strong signal from the back wall of the gall bladder where it adjoins the liver. Since this entire back surface is clearly shown, it may be considered as additional evidence that no calculi are present in the bladder. A calculus would cast a shadow similar to that produced by the glass rod in Fig. 4, *C*. The strong echo produced at the deep margin of the gall bladder is caused by the marked change in acoustic properties at the combined juncture of bile, bladder wall, and adipose tissue in the gall bladder bed. By contrast, Fig. 5, *C* shows a gall bladder which has a thick fibrous wall and is covered with an inflammatory exudate. This gall bladder is contracted and contains multiple, faceted, nonradiopaque calculi along with sandlike smaller stones. The cross-section Somagram is shown in Fig. 5, *D*. There is a strong surface echo from the thickened wall and very strong echoes from the calculi, since nearly all of the ultrasonic pulse is reflected back from the stones. The lower or deep surface of the gall bladder is only imperfectly seen due to the sonic shadow of the large calculi. In both of these gall bladder studies, a rough outline of the block of the liver included in the specimens can be seen. The echoes received from within the liver proper may correspond to the blood vessels and biliary radicals (probably where air or formalin solution is trapped within them).

The possibility of using ultrasonics for the localization of nonradiopaque foreign bodies has been recognized by several investigators.[20, 22, 25] In Fig. 6, *A*, three foreign bodies, a nail, a piece of wood, and a piece of plastic have been embedded in a block of liver. The identifying echoes from these three structures are of similar intensity, and are easily seen in the Somagram of Fig. 6, *B*. By x-ray, however, the latter two structures would be exceedingly hard to detect, especially in a thick structure such as the abdomen.

It was observed in our investigations that the pictures we have obtained from fresh specimens or from living subjects have been far superior to those obtained from formalin-fixed specimens. The hardened tissue apparently takes on different ultrasonic properties. We have seen metastatic adenocarcinoma and lymphosarcoma in fresh specimens of liver, but, after the specimens were fixed in formalin, we were unable to obtain satisfactory Somagrams for presentation in this paper. It is planned that the ultimate utilization of this instrument will be, of course, in the living subject.

A.



B.

Fig. 6.—The demonstration of foreign bodies. *A*, A nail, plastic rod, and match stick have been embedded deeply into the block of liver shown above, to demonstrate the ability of the ultrasonic unit to reveal foreign bodies in tissue. *B*, The strong signals from the foreign bodies are well seen within the liver tissue. The rear surface of the liver was shown by placing a flat cork sheet against it to increase the amplitude of the signal for identification purposes.

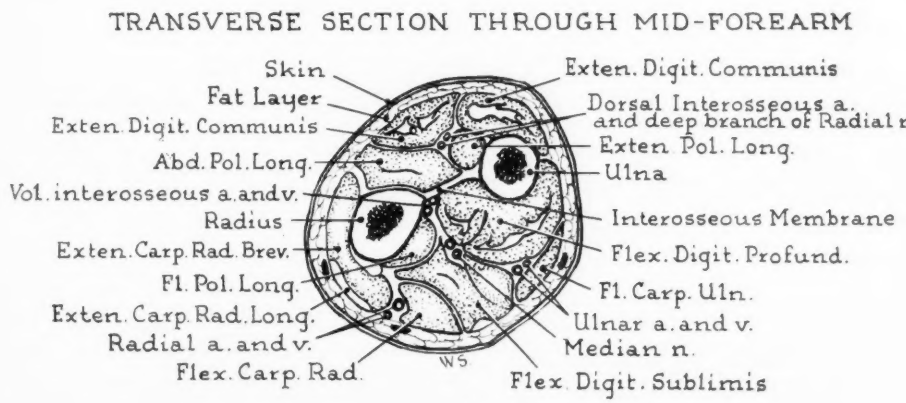
The Somagram shown in Fig. 7 was made of the posterior compartment of the forearm of one of the authors, and can be compared with the anatomic diagram of the midforearm shown above it. In the Somagram, both the ulna and the radius are very clear. The loss of all signals behind them is apparent. A faint signal may be seen from the lower and far side of the arm, where the beam penetrated the interosseous septum. The interosseous septum is well outlined and several of the intermuscular membranes are seen, including the fascial plane dividing the anterior and posterior compartment of the arm. The strong signals arising just below the skin surface represent the beginning formation of the superficial extensor tendons. The anterior compartment of the arm could be examined by turning the arm around.

DISCUSSION

The pulse echo system of measuring distance is not new. For many years it has been used by boats navigating at night or through fog. At intervals, a short blast from the ship's whistle is sent out. Echoes are then heard from islands or shorelines within range. By timing these echoes with a stop watch and noticing their direction, a plot is made and compared with the navigator's chart to find the exact position of the boat. During the first World War, Langevin and Cady devised an underwater system which sent out ultrasonic pulses and then detected echoes from underwater objects such as "U"-boats, for example. By knowing the direction from which the echo arrived and the time required for it to be received, the exact position of an object could be determined. This science is called sonar. During the last war, a similar system was developed—radar—which used radio wave pulses for the exact localization of planes and other objects at great distances.

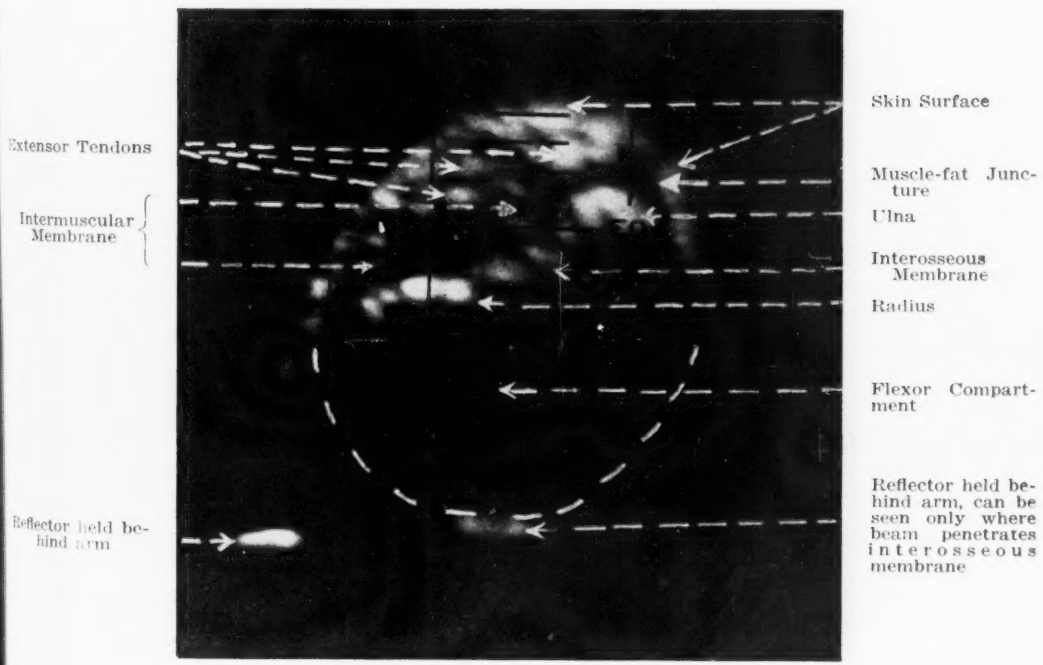
Dr. F. A. Firestone was the first to successfully apply the field of ultrasonics to materials testing by utilizing the microsecond techniques of radar and applying them to sonar-like transmitters to make a short-range, pulse-echo device. The Reflectoscope, as the instrument is called, is produced for industrial use in detecting flaws, grain size, thickness, and incipient fatigue in metal.[4, 11, 12, 13] It has been remarkably successful and has largely replaced x-ray testing in many applications. Its use in medical diagnosis was not very successful because of the mass of confusing echoes produced. The authors did extensive testing with this type of equipment on many kinds of tissue specimens in 1949.[29] Tissue interfaces and foreign bodies, etc., could be made to produce echoes, but the results were too erratic to be practical. Our results were in complete accord with the findings of Ludwig,[20] whose observations were published while we were completing our experiments. J. J. Wild has since reported somewhat better results[14, 15, 16, 27, 28] which may be due to his use of higher frequencies or to a narrower sound beam. Other groups have reported somewhat conflicting results in their attempts to apply ultrasonic reflection phenomena to diagnostic medicine.[2, 5, 6] However, no practical clinical application of the "Reflectoscope" is known to the authors. The therapeutic and pathologic effects of ultrasonics have been studied by large num-

bers of investigators, and this form of energy is now widely used therapeutically in Europe.[3, 17, 18, 21, 23, 24] The Somascope differs from other reflection instruments in several ways.* It produces a true picture of the object studied rather than a wave which is similar in appearance to the record pro-



A.

SOMAGRAM OF EXTENSOR COMPARTMENT OF MID-FOREARM



B.

Fig. 7.

*While this paper was in progress, J. J. Wild and J. M. Reed reported their results with a similar instrument. (Application of Echo Ranging Techniques to the Determination of Structure of Biological Tissues, Science **115**: 226, 1952.)

duced by an electroencephalograph. The sound pulse is confined to a narrow beam to improve definition. The crystal system employed is one with high efficiency and freedom from spurious vibrations.

The methods by which ultrasonics could be used to visualize tissue construction was made the subject of an extensive, theoretical study in 1948 by one of us,* and it was found that numerous methods exist. The results of this study will be published elsewhere. These methods all fall into one of two classes, either sonic reflection systems, like the Somascope, or through-transmission systems. In the last mentioned system, a transmitter of ultrasonics is placed on one side of the part to be examined and a receiver on the other side. Then, if the transmitter and receiver are caused to move synchronously and systematically over the part, the differences in the intensities of the received signal can be used to form a picture which is of a type similar to the roentgenogram in that it is principally dependent upon variations in the absorption of the beam for picture detail. Such a system was first proposed by Sokolov[25] as a method of materials testing. Dussik, Dussik, and Wyt,[7-10] in Germany, first applied this method to medicine. Their instrument is called the Hyperphonogram. Ballantine, Bolt, Heuter, and Ludwig,[1, 19] in this country, have improved the method, and are now further developing the instrument. Both groups have reported successful application of this type of equipment for roughly determining the size and outline of the cerebral ventricles. Other variations of the through-transmission system are possible,[2] but have found little application, and a technical analysis of these is beyond the scope of this paper.

There are many possible variations in instruments using the reflection system, but all of them are dependent upon the reflection of a sound wave by a discontinuity in the medium through which the wave is travelling. The two physical factors which are the most important in producing such a reflection are change in density and/or velocity of propagation of the wave. The definition of the picture obtained by such instruments is limited by the frequency used, so very high frequencies are desirable. The limiting factor is the increasing absorption of sound as the frequency is raised, with the result that a compromise between definition and depth of penetration must be made. The Somascope uses a simple 5,000 volt pulse of ⅓ microsecond duration applied to an X-cut quartz crystal. The pulse rate is not critical; 2,000 pulses per second are satisfactory. Two lenses and a concave reflector are used to provide adjustable focusing of the sound beam. The average sound power is not known accurately, but is in the order of a milliwatt.

One might speculate from our experience, to date, on the possible medical usefulness of this instrument. Certainly, it would be easy to visualize a soft tissue tumor in a cross-section Somagram of the arm, such as that shown in Fig. 7, or to pick up foreign bodies, as demonstrated in Fig. 6. Soft tissue pathology or organ size can probably be determined for organs close to the skin surface such as thyroid, salivary gland, and breast. Natural sonic contrasts, because of the fat layers, are of special value in visualizing these or-

*D. H. H.

gans, as well as deeper organs such as kidney, liver, and spleen. Fluid is also a good medium for sonic contrast, and should make possible visualization of the heart, urinary bladder, and the lung surface when fluid is present in the pleural cavity. In the case of pleural and abdominal fluids, it may be possible to differentiate between transudates and exudates, much as it was possible in Fig. 4, *B* and *C*, to differentiate between clear fluid and that containing talc. Bone and air pockets interpose a sonic barrier; therefore, some of the deep pelvic viscera and portions of other structures will prove more difficult to examine.

All the Somagrams presented in this article were made during the spring and summer of 1951. Since that time, we have been radically revising our equipment. At present, a new Somascope is nearly completed. Tests on this instrument have shown that it will have between 500 and 2,000 times the sensitivity and 10 times the definition of the unit reported here. This new unit will be tested clinically during the next few months. If, after a period of clinical testing on various structures and disease processes, the unit appears to be of practical use, the specific details of the improved unit will be published in appropriate journals so that other interested workers may independently evaluate the results obtained.

CONCLUSION

When properly applied, ultrasonic energy may be used to obtain echoes from tissue interfaces. These echoes can be made to generate a cross-section "picture" of the specimen. Ultrasonic pictures produced by this method are shown, along with pictures of the object studied. With technical improvements in the equipment and further information obtained from clinical studies, there should evolve an instrument of considerable value as an adjunct to x-ray in diagnosis.

We are indebted to Mr. Carl P. Spaulding for very helpful suggestions and aid during the initial theoretical phase of this development, to Dr. Dorothy A. Stott for technical assistance and encouragement in solving many problems during the past four years, to Mr. Gerald J. Posakony for the construction of the present transducer housing and mechanical sweep system, to Dr. Joseph H. Holmes for advice and aid in the preparation of this manuscript, to Dr. George I. Ogura, Dr. Emeric I. Dobos, and Dr. S. Prather Ashe for supplying pathologic specimens, and to Dr. Hans Jaffe of the Brush Development Company for supplying special ultrasonic crystals.

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CORRELATION OF A RAPID SLIDE-AGGLUTINATION TEST (CASTANEDA) WITH A TUBE-AGGLUTINATION TEST IN SCREENING SUSPECTED CASES OF HUMAN BRUCELLOSIS

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AS DISCUSSED elsewhere,[1] a precise diagnosis of brucellosis can be established only with the aid of laboratory procedures. An unequivocal diagnosis can be made when brucella organisms are obtained from a patient. However, even when cultural techniques are exploited to their full advantage, organisms are often not isolated from the body fluids of the patients. Furthermore, the widespread use of antibiotics having antibrucella activity in patients with fever of doubtful etiology frequently destroys the bacteriologic evidence of infection but still leaves febrile patients with an undiagnosed illness. For these reasons the agglutination test is assuming more and more importance in the diagnosis of suspected cases of brucellosis.

The tube-agglutination reaction is a dependable test for the diagnosis of suspected cases of brucellosis when interpreted along with epidemiologic information, the manifestations of the illness, and other laboratory data such as the total leukocyte and differential counts.[1-3] The successful application of the agglutination reaction is dependent upon the proper selection of antigen and the technique employed in performing the test. In attempting to arrive at a diagnosis of brucellosis in an ambulatory patient, or even in hospitalized individuals, considerable time is often expended waiting for the results of the agglutination test. From the time the serum is mixed with antigen a minimum of twenty-four hours of incubation is desirable before the test can be read. While the tube-dilution test has been relied upon in this clinic for several years for suspected cases of brucellosis, attempts have been made to speed up the interpretation of a patient's illness with a rapid slide test. Attention was first turned to the rapid slide-agglutination test as recommended by Huddleson.[4] In this clinic, Agnew[5] attempted to correlate the rapid slide test with other laboratory procedures, such as the tube-agglutination test, complement fixation test, and blood cultures. Antigen for the slide test was supplied by Dr. Huddleson. Unfortunately, the results of the rapid slide test did not correlate too well with those of the tube-dilution technique. Too many false-positive tests were encountered with the slide method.

In 1947, Dr. M. Ruiz Castaneda of Mexico City visited this clinic and demonstrated a rapid slide test with an antigen that he had prepared in his laboratory. Preliminary comparative tests with this slide test and the tube test, in which the standard *Brucella abortus* antigen of the Bureau of Animal Industry

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was used for the tube test, indicated a high degree of correlation between the two procedures. The Castaneda antigen appeared to possess several advantages, the chief of which was that it had been titrated against the tube technique so that clumping of the antigen usually took place on the slide with serum that possessed a titer of 1 to 100 or above by the tube test. Except in a few instances, the slide test showed no agglutination with a serum possessing demonstrable agglutinins with the tube test in a titer of 1 to 20 and 1 to 40. This feature was highly desirable in a reliable screening test because a considerable number of healthy individuals in Minnesota and adjacent areas have apparently been exposed to brucella organisms and possess low titers of brucella agglutinins. However, less than two per cent in one study had titers of 1 to 160 or above.[6] Furthermore, over 90 per cent of the patients with bacteriologically proved brucellosis had titers of 1 to 320 and above. It appeared, then, if a suspected person with brucellosis had a positive slide test with the Castaneda antigen, it meant that the routine tube-agglutination test would yield a titer of 1 to 100 or higher. Further advantages of the Castaneda test were that it could be performed at the bedside or in the clinic with a drop of whole blood from the patient's finger or ear lobe, and the test could be read within sixty seconds.

The test has now been correlated in this clinic with the tube-dilution agglutination reaction during the years 1947 to 1952. Simultaneous tests have been performed on over a thousand specimens of blood from an equivalent number of human subjects. Because the slide test has proved to be reliable and of considerable aid in the rapid screening of suspected cases of brucellosis, the results are being reported at this time. The technique for preparing the antigen is also being supplied since the only available published statement has been presented in Spanish by Castaneda[7] in a monograph on brucellosis, which is now out of print.

PREPARATION OF THE ANTIGEN

The methods for preparing the antigen have been supplied by Dr. Castaneda. Thirty Roux bottles containing liver infusion agar are inoculated with a smooth culture of *Br. abortus* and incubation is carried out for forty-eight hours at 37° C. The bacterial growth is washed off with sterile, physiologic saline solution and filtered through cotton. About 800 ml. of saline are utilized for this purpose. Approximately 80 ml. of formaldehyde solution (40 per cent) are added to the bacterial suspension so that the final concentration of formaldehyde is 10 per cent. The mixture is then permitted to stand at room temperature for twenty-four hours. The material is then centrifuged in an angle centrifuge, the sediment resuspended in isotonic saline solution, and standardized to a turbidity of 2 ml. (Gates). At this turbidity, which approaches the concentration of the finally prepared antigen, the bacterial growth from 30 bottles will yield 100 ml. of material. At least three batches of 30 bottles each are gathered for staining and standardization making a total of 300 ml. of antigen. The antigen is stained by adding an aqueous solution of 1 per cent methylene blue (Hartman-Leddon Co. with a dye content of 84 per cent). About 60 ml. of methylene blue solution are required for the proper staining of 300 ml. of bacterial suspension. Approximation of the desired color can be made by comparing with a standardized antigen.* A very deep blue color is desirable. The stained preparation is permitted to stand at room temperature for twenty-four hours and then centrifuged. The resulting stained sediment obtained from 300 ml. of bacterial suspension is then resuspended in about 200 ml. of a 1.1 per cent

*Standardized antigen may be obtained from Dr. M. Ruiz Castaneda, Investigaciones Medicas, Hospital General, Mexico, D. F.

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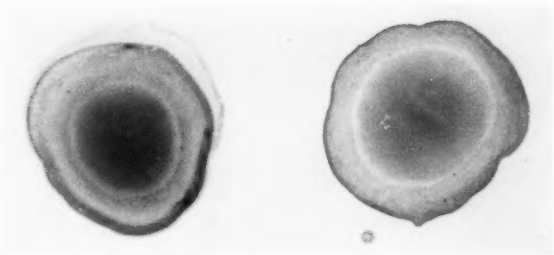

Fig. 1.—Photograph of rapid slide-agglutination tests: on the left, a typical negative reaction is noted. On the right, a positive reaction is illustrated with the blue-green ring around the periphery, representing agglutinated and stained antigen. The positive reaction appeared within sixty seconds after mixing the whole blood and antigen.

solution of sodium citrate containing merthiolate in a concentration of 1:10,000. This more concentrated suspension will permit a sufficient range of dilution when final titration is carried out.

Standardization of the antigen is quite important with respect to the color and to the reaction with serum containing agglutinins. Comparative tests are made with a standard antigen. The primary object of the standardization is to obtain a positive slide test only when antibody is present in a titer of 1:100 or above. Standardization is carried out by making a series of dilutions with a serum known to have brucella agglutinins. Castaneda states that a drop of standard antigen is then added with a 4 mm. wire loop to each of several slides, and a drop of each of the diluted sera is added to the antigen with a 3 mm. wire loop and mixing carried out. Margination of the agglutinated particles is favored by gently rotating the slide. The last two dilutions giving agglutination are selected for comparison with the various samples of the unstandardized antigen. These samples are prepared by adding 1 ml. of antigen to 0.1 ml. saline, 1 ml. + 0.2 ml., 1 ml. + 0.3 ml., etc. That sample which on repeated tests reacts with the same intensity as the standard antigen is selected as a guide for the final step in standardization. This final step calls for the diluting of the standard and unknown antigens 1:50 and then performing comparative tube-agglutination tests with sera of titers known to range from 1:160 to 1:640. Any discrepancies should be noted. In addition, several comparative slide tests should be carried out with the standard and the newly prepared antigen with various samples of bloods containing agglutinins. If the antigen is too sensitive, it has been diluted too much, and must be concentrated. This is carried out by centrifuging the bacterial suspension, and then resuspending the sediment in the proper volume. A relatively insensitive antigen may be due to the use of an unfavorable culture, to a concentrated antigen, or to insufficient coloring with methylene blue.*

PERFORMANCE OF THE SLIDE TEST

The test with whole blood may be carried out with citrated blood, or blood taken directly from a finger tip or ear lobe. A drop of antigen is placed on a clean slide with the aid of a wire loop having a diameter of 4 mm. A drop of blood is then taken up in the wire loop and mixed with the antigen. The mixture is extended to a circular area of about 1.5 cm. in diameter. When the slide is inclined slightly the blood-antigen mixture should accumulate at the lower side of the circle. By manipulating the slide gently the fluid should be made to rotate around the periphery of the circle. Within thirty to sixty seconds of rotation, the presence of enough brucella antibody establishes a striking blue ring at the periphery with a reddish center, which results from the separation of the erythrocytes from the agglutinated antigen. The speed and intensity of the reaction are usually related directly to the amount of antibody present in the blood. In a negative reaction, antibody is either absent or present in a concentration too low to detect, and a reddish ring is formed by the accumulation of erythrocytes at the periphery, which surrounds a background of uniform greenish mixture. Often the red cells agglutinate, leaving a blue center, which is due to liberated antigen. Fig. 1 is a photograph of a positive and negative reaction.

THE TUBE-AGGLUTINATION TEST

The antigen and technique used in the tube-agglutination test have been employed in this laboratory for several years. The antigen has been prepared from

*According to Castaneda, the standard antigen has been standardized by two tests: (1) A dilution of 1.50 will produce a tube-agglutination test one dilution below the titer obtained with a phenolized antigen prepared with the same abortus strain (this tube antigen is less sensitive than the antigen employed in the present study). (2) The antigen is also titrated according to Huddleson's technique with sera of low titer.

a smooth culture of *Br. abortus* by the Bureau of Animal Industry of the United States Department of Agriculture. Comparative observations with other antigens have demonstrated that this antigen is a sensitive and satisfactory preparation. It is equally applicable to the sera from patients having infections due to any three of the brucella species. The test is performed as follows: 10 tubes (13 ×100 mm.) for each serum tested are placed in a rack with 0.9 ml. of physiologic saline solution being added to the first tube, and 0.5 ml. to the remaining nine tubes. With a serologic pipette of 1 ml. capacity and calibrated to 0.01 ml., 0.1 ml. of the serum to be tested is added to the first tube. The mixture of saline solution and serum is drawn up and expelled from the pipette seven or eight times. Then 0.5 ml. is transferred from the first to the second to the third tube, and so on down through the tenth tube. An additional tube containing only 0.5 ml. of saline solution is used as a control. To each tube is added 0.5 ml. of antigen. The contents in each tube are mixed and incubation is carried out in a water bath at 37° C. for 48 hours. The end point is that tube with the highest dilution of serum showing complete clumping of the antigen in the bottom of the tube and a clear supernatant. The first tube in the rack represents a dilution of serum of 1 to 20, the second 1 to 40, and so on up to the last tube, which is 1 to 10,240. The higher dilutions of serum are essential in order to circumvent the occasional specimen displaying a prozone phenomenon in which agglutination may not take place in dilutions up to 1 to 160 or 1 to 320.

COMPARATIVE RESULTS OF THE SLIDE-AGGLUTINATION TEST WITH THE TUBE-DILUTION METHOD

Two general studies were carried out, involving 1,082 specimens of blood. In the first series of observations, a correlation of the two tests was made with 746 specimens of blood obtained from donors appearing at the blood bank of the University Hospitals. As pointed out elsewhere,[7] a significant number of these individuals had demonstrable brucella agglutinins. In performing the slide test with the bloods from the donors, serum was used. In the second series comparative tests were performed with 336 specimens of blood from suspected cases of brucellosis. In this group whole blood was employed for the test, using either blood obtained directly from the ear lobe or a finger, or fresh, oxalated venous blood. The results of the slide test when correlated with the tube-dilution test are shown in Table I. A positive slide test was obtained with 131 of the 1,082 specimens of blood, while 330 of the bloods revealed brucella agglutinins with the tube-dilution technique. There were two specimens that showed a positive slide test, but agglutinins could not be demonstrated by the tube-dilution method. This discrepancy probably represents the phenomenon of "blocking antibodies" masking the results of the tube-dilution method. Castaneda[8] has emphasized that the slide technique will detect the presence of "blocking antibodies," which obscure the results of the tube-dilution method. The significance of "blocking antibodies" will be discussed shortly.

An examination of these results indicates that the slide test yields few false-positive tests when compared to the tube-dilution agglutination method. On the other hand, a significant titer of agglutinins was detected with the tube-dilution

technique in a number of bloods that showed a negative slide test. This was particularly apparent in the bloods that yielded a titer of 1 to 160, in which 13 of 33 specimens gave negative slide reactions. As the titer of the agglutinins progressively increased by the tube-dilution method, there was a corresponding closer correlation with the slide method.

An attempt was also made to quantitate the results of the slide-agglutination test with the titer anticipated with the tube-dilution technique. This was done by noting the speed with which clumping of the stained brucella cells took place after the initial mixing of the whole blood or serum and the antigen, and by the intensity of the blue-green ring formed by the agglutinated bacteria. A 0 reaction meant that no clumping of the bacterial cells was demonstrable and a blue ring was not formed. A ± reaction indicated a faint blue ring that took more than sixty seconds to form. A 1+ test was a blue ring that formed within thirty to sixty seconds. A 2+ test designated a blue ring that appeared after ten to thirty seconds. A 3+ test indicated the presence of a ring within ten seconds, and a 4+ test designated the immediate appearance of the blue ring upon bringing the blood and antigen together. The correlation of the quantitative slide test with the quantitative tube test is presented in Table II. It is to be noted that there is a rough correlation of the 131 positive slide tests with the tube-dilution tests. The blue ring formed in a positive slide test was particularly intense and appeared more quickly with those specimens of blood that contained the higher titer of agglutinins by the tube-dilution method. A positive slide test was obtained with only three specimens of blood when the titer with the tube-dilution method was 1 to 40 or lower. The present results with the slide agglutination test indicate quite clearly that the clinicians were not handicapped by the appearance of false-positive reactions. But a significant number of negative slide tests were obtained in patients with active disease who had a titer of 1 to 160 and above with the tube-dilution test. It is to be emphasized that two different antigens from two different sources were being compared, and the maximum titer of agglutinins in the tube-dilution method was sought by incubating the antigen-blood mixtures for 37° C. for forty-eight hours.

DISCUSSION

This investigation has demonstrated a fairly good correlation between the results of the rapid slide-agglutination test and the tube-agglutination test in detecting suspected cases of brucellosis. Previous experience in this clinic with other antigens for the rapid slide test had led us to discard the rapid method because too many false-positive reactions were encountered. In the present study with the Castaneda antigen only two specimens of blood yielded a positive slide test when the tube-agglutination test was negative. The reliability of a positive reaction indicating that agglutinins would be detected by the tube-dilution technique saved considerable time in evaluating the clinical status of patients and getting treatment under way, prior to the clinicians learning about the results of the tube-agglutination test. On the other hand, there were several instances when a significant titer of brucella agglutinins was detected by the tube-dilution method, and the slide test remained negative. This discrepancy should continue

TABLE I. CORRELATION OF SIMULTANEOUS SLIDE-AGGLUTINATION TESTS AND TUBE-DILUTION TESTS IN 1,082 SPECIMENS OF BLOOD

	RECIPROCAL OF TITER OF BRUCELLA AGGLUTININS WITH TUBE-DILUTION METHOD											
	LESS THAN 20	20	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480
Number of positive tube-dilution tests in relation to titer	752	68	73	47	33	28	26	21	15	16	2	1
Number of positive slide tests in relation to titer of tube test	2	0	1	7	20	23	24	20	15	16	2	1

TABLE II. AN ATTEMPT TO QUANTITATE THE SLIDE TEST AND CORRELATE THE RESULTS WITH THE TUBE-DILUTION AGGLUTINATION TEST IN 1,082 SPECIMENS OF BLOOD

RESULTS OF SLIDE TEST	RECIPROCAL OF TITER OF BRUCELLA AGGLUTININS WITH TUBE-DILUTION METHOD WITH CORRESPONDING NUMBER OF POSITIVE SLIDE TESTS											
	BELOW 20	20	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480
0—No reaction	750	68	72	40	13	5	2	1				
±—Faint blue ring in 60 seconds	2			1	1		1	1				
1+—Blue ring in 30 to 60 seconds			1	3	7	4	4	2				
2+—Blue ring in 10 to 30 seconds				1	8	13	9			3		
3+—Blue ring in 10 seconds				1	4	6	5	4	7	4	2	
4+—Blue ring formed immediately				1			5	13	8	9		1

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to emphasize the necessity of performing a tube-dilution agglutination test with the serum of *every* suspected case of brucellosis. Perhaps a closer correlation would have resulted in the present study if the same source of antigen had been used in both tests.

Castaneda has stated that the slide-agglutination test as described in this report can elicit a positive reaction in an occasional specimen of blood that yields no agglutinins with the tube method because of the presence of "blocking antibodies." This may explain the results in those two instances in which a positive slide test was obtained but no agglutinins were demonstrated with the tube technique. From a practical viewpoint, however, "blocking antibodies" are present in such low titers that this phenomenon need give rise to no confusion in the diagnosis of brucellosis.[9]

Further investigations should be carried out in other clinics and laboratories with the type of antigen utilized for the rapid slide-agglutination test in this study. The results should be correlated with the tube-dilution agglutination method in which antigen from the same source should be used in the two tests.

SUMMARY

1. A brucella antigen (Castaneda) prepared for a rapid slide-agglutination test has been correlated over a five-year period with the tube-dilution agglutination technique. Simultaneous tests were performed with 1,082 specimens of blood.

2. The advantages of the rapid slide antigen were that it had been titrated in a manner so that positive reactions were only occasionally encountered with bloods containing a low titer of agglutinins, and few false-positive reactions were observed.

3. While the rapid slide-agglutination antigen employed in the present study was of considerable aid to the clinicians in screening suspected cases of brucellosis, the simultaneous use of a quantitative tube-dilution agglutination technique emphasized the necessity for the latter test in every case. In addition, cultural methods should also be available.

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LABORATORY METHODS

THE DETERMINATION OF FLUORIDE IN DRINKING WATER AND BIOLOGICAL MATERIALS

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THE more widespread use of fluoride compounds by physicians and dentists has caused considerable interest in the accumulation and elimination of this element in body fluids and tissues. We found the evaluation of fluoride content in water, biological materials, and a wide variety of edible products needs to be extended. Many of the determinations for fluoride found in the literature were tedious and not too accurate. Therefore, we have tried to devise a simpler and more accurate fluoride analysis, which is presented here.

Apparently, drinking water is the most common and the major source of fluorides. Fluoride in the ratio of 1:1,000,000 in the water supply is considered enough for prevention of dental caries with a minimal occurrence of mottled enamel.[1] In foodstuffs fluorides appear in small amounts.[2] It is important to determine fluoride in districts where the natural water supply contains the element close to the maximal requirement, so that no additional fluoride will be added to the water, which would cause extreme mottling of tooth enamel. Greenwood[3] states that when fluoride is added to the diet in small amounts, it is excreted in proportion to the intake. Individuals on a normal diet containing no added fluoride excrete about 0.3 to 0.5 mg. per day; this presumably represents the amount ordinarily present in the diet. It is known that in certain parts of the world the soil contains sufficient soluble fluoride so that the drinking water and the food grown on the soil contain enhanced amounts of fluoride relative to other localities. Individuals living in such communities are prone to develop mottled enamel or dental fluorosis, an unsightly condition of the teeth. At the same time the incidence of dental caries in these communities is in general lower than elsewhere.

Fluoride is stored, in the form of insoluble calcium fluoride, in the liver and skin, and especially in the bones and teeth. It impairs the nutrition, renders the osseous structure more brittle, and mottles the clear pearly enamel of the teeth with dull white areas.

As the interest in fluoride compounds increases, it is essential to find an analysis for the fluoride ion which is not only rapid and convenient, but which

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also gives an accurate and precise determination for a wide range of organic and inorganic substances. Several methods were found in the literature which appeared to meet these qualifications.

The fluoride ion can be titrated in neutral (phenolphthalein) aqueous solution to the methyl red end point with thorium nitrate,[4] aluminum nitrate,[5] or cerous nitrate.[6] When these three methods were tried, the end points were not reproducible.

A method involving the formation of the complex FeF_6^{-3} did not give reliable results.[7, 8]

The procedure, developed by Saylor and co-workers,[9, 10] of titrating fluoride in a slightly basic solution at 95° C. with aluminum chloride and Eriochromcyanin R indicator did not give the precision expected.

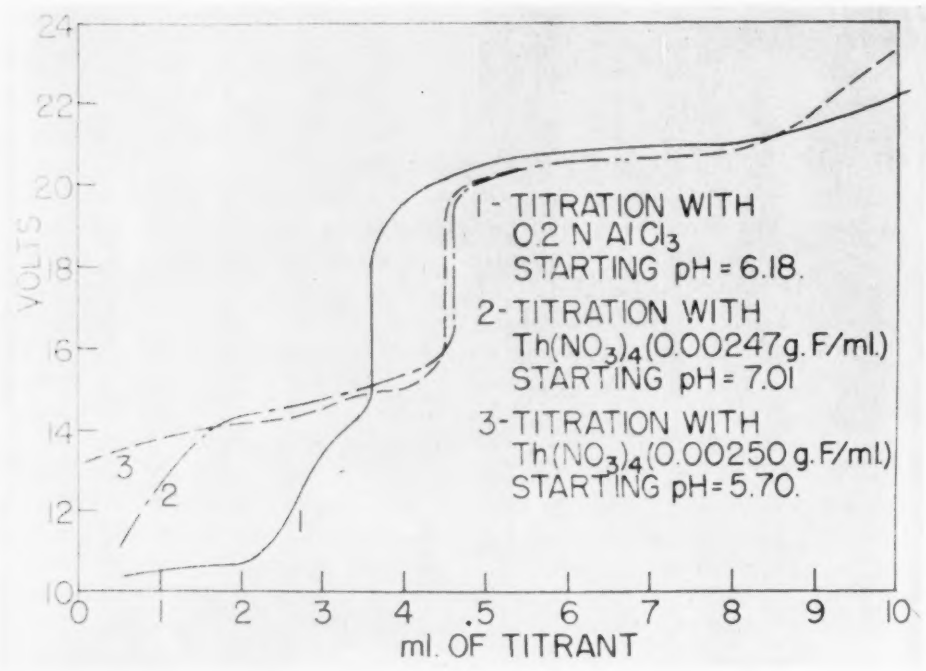


Fig. 1.—Potentiometric titration of 25.0 mg. NaF with $Th(NO_3)_4$ and $AlCl_3$.

Potentiometric titrations with ferric chloride or barium chloride as titrants gave indistinct results and no end point. Accurate, reproducible end points were obtained with a glass electrode system, when aluminum chloride or thorium nitrate was added to a neutral fluoride solution (Fig. 1).

Smith and Gardner[11] report a reproducible, accurate method, suitable for routine determination of fluoride in blood. The procedure requires a preliminary distillation from concentrated sulfuric acid at 134° to 137° C., evaporation and ashing of the distillate in the presence of lime, and a final redistillation

from perchloric acid. The fluoride content of a suitable aliquot of the distillate is then measured by the modified Williams titration.[12] Our method requires one distillation and no previous ashing or redistillation.

In the titration of fluoride with thorium nitrate and sodium alizarin sulfonate indicator, others[13-16] have adjusted the acidity with dilute hydrochloric acid and sodium hydroxide in addition to a buffer of sodium monochloroacetate. The color during this titration is pale, and the change at the end point is difficult to judge.

In this laboratory the acidity of the fluoride solution was adjusted with ammonium hydroxide and acetic acid, thus eliminating the unstable sodium monochloroacetate buffer, since the ammonium acetate formed in the solution acts as a satisfactory buffer. The solution can be titrated accurately with standard thorium nitrate to the sodium alizarin sulfonate, or as we used, the Chrome Azurol S end point.

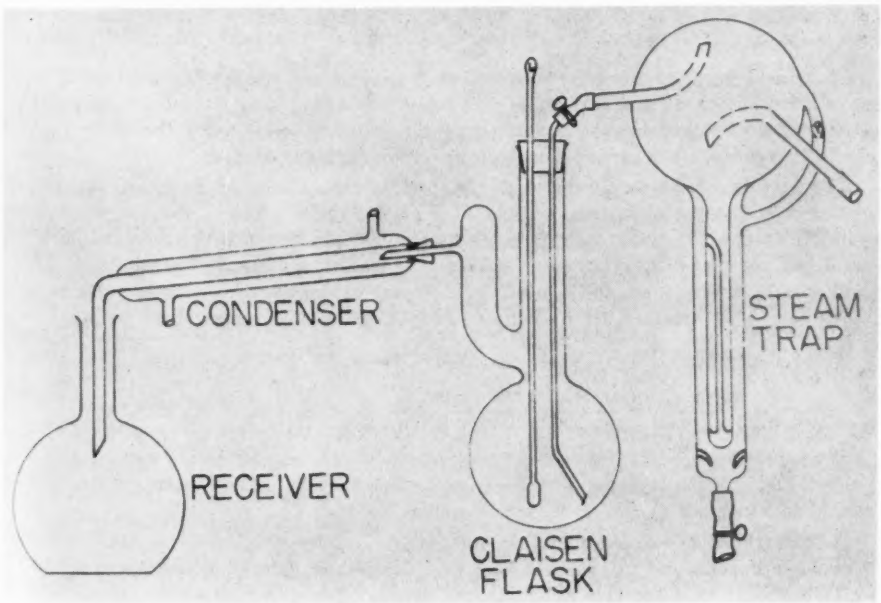


Fig. 2.—Distillation apparatus.

Willard and Horton[17] state that purpurin sulfonate, Alizarin Red S, Eriochromeyanin R, dicyanoquinizarin, and Chrome Azurol S are even better two-color indicators than sodium alizarin sulfonate. Although we found no substantial difference among these five indicators, it was most convenient for us to use Chrome Azurol S.

APPARATUS AND REAGENTS

(a) A Parr peroxide bomb (Parr Instrument Co., Moline, Ill.) was used to decompose the organic fluoride materials.

(b) The steam distillation apparatus, shown in Fig. 2, consists of a modified 250-ml. Claisen flask, condenser, thermometer, and steam trap.

(c) The potentiometric titrations were made with the aid of a Beckman Model G pH meter, glass electrode system, mechanical glass stirrer, and a 10-ml. burette.

(d) All the distilled water used in making solutions and used for wash water was boiled to remove the carbon dioxide, since it was found that carbon dioxide seriously interfered with the titration.

(e) Approximately 17.5 Gm. of thorium nitrate tetrahydrate was dissolved and made up to 1 liter in 0.05M acetic acid. Thorium nitrate is more stable in the dilute acid than in water. This solution was standardized against the following sodium fluoride solution.

(f) About 0.2 Gm. of sodium fluoride (C. P., dried at 110° C.) was weighed quantitatively, dissolved in water, and diluted to 200 ml. This solution must be made fresh each time that the thorium nitrate is standardized because the reagent reacts with the glass container.

(g) The indicator was a 0.05 per cent aqueous solution of Chrome Azurol S. which should be freshly prepared about every four weeks.

METHOD

Titration.—In order to standardize the thorium nitrate, a 25-ml. aliquot of sodium fluoride solution with 8 to 10 drops of 0.05 per cent Chrome Azurol S is adjusted to about pH 3.5 in the following manner:

Three or 4 drops of 2 per cent ammonium hydroxide are added. The solution is now a green-yellow color. A 0.5 per cent acetic acid solution is added until the solution is just pink, then one drop of ammonium hydroxide turns the solution yellow, and the desired pH is reached. This procedure is carried out carefully with a medicine dropper.

The fluoride solution is titrated with the thorium nitrate solution from a 10-ml. burette. The color quickly turns pink, then a slight blue precipitate is noted as the end point is approached. At this point the flask is swirled carefully and the titration is done slowly in order to find the point of maximum color change or maximum amount of precipitate. This is the end point. It is best to take several readings just before the end point in order to avoid overtitration. The thorium nitrate should be restandardized about every four weeks.

Standardization Calculation:

$$\frac{\text{at. wt. F} \times \text{Gm. NaF} \times \text{ml. aliquot}}{\text{mol. wt. NaF} \times \text{orig. vol. NaF soln.} \times \text{ml. Th}(NO_3)_4} = \text{Gm. F/ml. Th}(NO_3)_4$$

Decomposition.—Nichrome wire is properly fastened on the inside cap of the Parr peroxide bomb. To the inside cup of the bomb is added 1 Gm. of potassium nitrate, 0.5 Gm. of starch, and 1 dipper (1 cm. by 2 cm.) of sodium peroxide.

A 0.5 to 1.0-Gm. sample containing fluoride is carefully weighed in an aluminum dish, if the sample is solid and dry such as bone ash, dentine, or enamel, then transferred to the cup of the bomb. If the sample is liquid, such as whole blood, serum, plasma, urine, or cerebrospinal fluid, it is weighed into a previously weighed gelatin capsule. The gelatin capsule is help upright by placing it in the hole of a cork stopper. The liquid sample is placed in the capsule by a dropper. The sample is transferred to the bomb. The inside cap is set in place and the bomb head tightened. If the sample is liquid, the bomb is not shaken. But if it is solid, the bomb is shaken to mix the contents well. The bomb is set in a water-cooled firing container, fired behind a shield of safety glass, then allowed to cool.

The head of the Parr bomb is carefully removed and washed with a small stream of warm water into a 250-ml. beaker. The inside cup is removed and placed in the beaker by means of tongs. The beaker is covered with a watch glass. Warm water is poured under the watch glass until it just covers the bomb cup.

Distillation.—After the effervescence subsides and the decomposition is complete, the mixture in the beaker is transferred quantitatively to a 250-ml. modified Claisen flask containing 8 to 10 glass boiling beads (Fig. 2). This flask should be pretreated with boiling 1:1 sulfuric acid-water mixture in order to prevent the formation of nonvolatile silicon

oxyfluoride, $SiOF_2$, which is found in the presence of hydrated silica.[18] The flask is closed with a two-hole rubber stopper, through which passes a thermometer and a glass tube extending to the bottom of the flask. The condenser is attached, and 75 ml. of 1:1 sulfuric acid-water mixture is slowly and carefully added through the glass tube with the aid of a small funnel attached to a short length of rubber tubing.

Slowly the contents of the distilling flask are heated and liquid is distilled until the boiling point reaches 130° to 135° C. Then steam is admitted through the long glass tubing, and the distillation is continued with the temperature maintained close to 135° C. until 250 to 300 ml. of distillate is collected. The silicon tetrafluoride, which distills, reacts with steam in the condenser to produce a distillate containing fluosilicic acid.

The volume of the distillate must be known, therefore it may be transferred to a suitable volumetric flask and made up to the mark, or the original volume may be measured very carefully by use of a graduated distillation receiver. Usually a 100-ml. aliquot of the fluoride solution (an aliquot representing about 0.5 mg. fluoride) is removed and placed in a 250-ml. Erlenmeyer flask for titration. About 10 drops of 0.05 per cent aqueous Chrome Azurol S indicator is added from a dropper. The acidity of the solution is adjusted, using ammonium hydroxide and acetic acid, in the same manner as for the standardization of thorium nitrate. The remainder of the titration is the same as for the sodium fluoride-thorium nitrate.

Calculation of Per Cent Fluoride in the Sample:

$$\frac{\text{ml. } Th(NO_3)_4 \times \text{Gm. F/ml. } Th(NO_3)_4 \times \text{total ml. of distillate} \times 100}{\text{sample wt.} \times \text{ml. aliquot titrated}} = \text{per cent fluoride in the sample.}$$

RESULTS

The analysis of several known compounds is shown in Table I. The highest average deviation from the calculated per cent fluoride is 0.04 per cent, which indicates that if the analysis is carried out properly, the error is not too great. The analysis of tooth dentine and enamel and a few foods corresponds fairly well to that found by previous workers[2, 21, 22] using other more tedious methods of analysis.

Hill and co-workers[23] studied the fluoride content in some communal water supplies in the United States. They found fairly high concentrations of fluoride in the waters of Ohio, Indiana, and Illinois. We found that Dayton city water contains practically no fluoride.

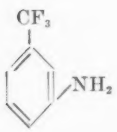
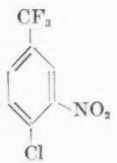
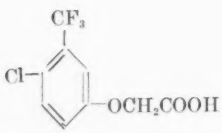
DISCUSSION

The fluorides in drinking water may be titrated directly with thorium nitrate and Chrome Azurol S without distillation. If the amount of fluoride in the aliquot to be titrated is less than 0.5 mg., it was found that if a 50-ml. aliquot is taken and 50 ml. of 95 per cent ethanol is added, the titration reaction is stoichiometric.

The procedure described eliminates the evaporation of the distillate and washing in the presence of lime, and a final redistillation from perchloric acid as outlined in the procedure by Smith and Gardner.[11]

All the experimental biological materials and organic compounds used in this work completely decomposed in the Parr bomb. It might be necessary to run a blank on the reagents, since it is known that some samples of C. P. sulfuric acid contain traces of fluoride.

TABLE I. ANALYSIS OF COMPOUNDS AND MATERIALS CONTAINING FLUORIDE

COMPOUND OR SUBSTANCE	FOUND PER CENT F	CALC. PER CENT F	AV. DEVIATION
NaF	45.24 45.22 45.24	45.24	0.01
NH_4HF_2	66.65 66.64 66.68	66.67	0.03
$Ca(C_2F_3O_2)_2$	42.86 42.81 42.85	42.84	0.02
$F_5C_4O\,C_4F_5$	75.28 75.35 75.35	75.33	0.03
	39.00 39.03 38.98	39.01	0.02
	31.55 31.58 31.56	31.57	0.02
	35.36 35.44 35.43	35.40	0.04
	25.30 25.26 25.27	25.28	0.02
	22.41 22.38 22.42	22.40	0.02

	Found per cent F		Found per cent F
Normal enamel (dry)	0.0099 0.0116 0.0122	Mottled enamel (dry)	0.0232 0.0400 0.0368
Normal dentine (dry)	0.0178 0.0157 0.0162	Mottled dentine (dry)	0.0382 0.0426 0.0389
Crest of 36-year-old illeum (dry)	0.0223 0.0199 0.0228	Milk (dry)	0.0000 0.0000 0.0000
Potatoes (dry)	0.0001 0.0000 0.0001	Pablum	0.0012 0.0009 0.0018
Dayton city H_2O	0.0000 0.0000 0.0000	Normal whole blood	0.0001 0.0001 0.0002

Rowley and Churchill[15] carried out the distillation at 160° to 165° C. and collected 350 ml. of distillate. A temperature of 135° C. is high enough, and it is not necessary to collect more than 250 to 300 ml. of distillate. Approximately 40 per cent of the total amount of fluoride was found to distill over in the first 150 ml. before the temperature of the solution in the flask had reached 130° C. As soon as steam was admitted, about 60 per cent of the fluoride distilled over in 50 ml. of distillate. The following 50 ml. had a very small amount of fluoride, and the next 100 ml. contained none that was detectable by titrating with thorium nitrate.

Results were better if the distillation was not carried out too fast, thus eliminating traces of sulfuric acid being carried over into the distillate caused by splashing or foaming. Sulfate ions greatly interfere with the titration as does any ion that forms a precipitate or a complex with fluoride or thorium.

It is now generally known that the pH of the titration must be controlled in order to obtain a sharp end point with the use of an adsorption indicator. This acidity control restricts the ionization of thorium fluoride. As the acidity decreases, the amount of fluoride ion in solution increases, probably because of the precipitation of basic thorium salts or hydrous thorium oxide.

When the solution is buffered before titration, the fluosilicate ions, produced by the action of steam on the silicon tetrafluoride, will be hydrolyzed to produce meta silicic acid and fluoride ions. Therefore at higher pH values the proportion of fluoride ions will be greater. Assuming that the fluosilicate ion does not form a slightly ionized compound with thorium, it is then essential to find a suitable pH range to equalize these two opposing reactions in the same system.

Malaprade[19] showed that the neutralization of fluosilicic acid takes place in two stages:

$$H_2SiF_6 + 2\ NaOH \rightleftharpoons Na_2SiF + 2\ H_2O \qquad (1)$$
$$Na_2SiF_6 + 4\ NaOH \rightleftharpoons 6\ NaF + SiO_2 + 2\ H_2O \qquad (2)$$

He points out that fluosilicate ion occurs in appreciable proportions only in solution with a pH below 3. If it is desired to isolate fluoride or silica quantitatively from the fluosilicate, the pH must be greater than this value.

The titration of fluoride solution above pH 4 with thorium nitrate is unsatisfactory, because at that acidity a build-up to the proper intensity of color in the adsorption indicator takes place.

The optimum acidity found for this titration was pH 3.0 to 3.6. In our work we found that changes in concentration of buffer had only a slight effect on the pH of the titrated solution. Neutralization of the acid solution of fluoride-fluosilicate ions was best done with ammonium hydroxide, since ammonium ions were found to interfere less with the titration than sodium ions from sodium hydroxide. Hydrochloric acid had too strong an effect on the acidity, and since chloride ions also interfere, acetic acid, a weaker acid with no serious effects on the titration, was highly satisfactory for the controlling of pH. No additional buffer is needed with the ammonium hydroxide-acetic acid system.

The use of boiled distilled water to eliminate carbon dioxide in all titrating and titrated solutions gave a greater accuracy in the results.

As would be expected, a marked break in the potentiometric curve was observed when sodium fluoride was titrated with thorium nitrate (Fig. 2). Aluminum chloride also gave accurate and reproducible results. The solution for the potentiometric titration was prepared in the same manner as for the adsorption indicator titration. It was found that a sharper break in potential was obtained if the fluoride solution was close to pH 7. This method for fluoride analysis is not recommended for routine use as the fluoride ion does react with the thin glass membrane of the electrode.[20] However, it is by far the most precise and accurate method for very small amounts of fluoride.

SUMMARY

This work describes an analysis for large or small amounts of fluoride in organic and inorganic compounds, drinking water, foods, and biological materials.

The method makes use of the Parr bomb in decomposing the sample, a steam distillation, and a titration with a standard thorium nitrate solution with Chrome Azurol S as the absorption indicator.

Mottled enamel and dentine were found to have slightly more fluoride present than in normal teeth. Pablum was found to be quite high in fluoride content. Dayton city water contains less than one ten-thousandth per cent fluoride.

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A MICROMETHOD FOR SERUM BILIRUBIN

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THE importance of serum bilirubin levels in the clinical management of infants with erythroblastosis fetalis[1-4] necessitates a rapid micromethod for the determination of serum bilirubins using capillary blood.

Malloy and Evelyn[5] described the measurement of "direct" and "indirect" bilirubin content of serum with the Evelyn photoelectric colorimeter using a diazo reagent. Ducci and Watson[6] modified the method by measuring "one minute prompt direct" and "total" bilirubin content of serum. The micromodification of the above methods requires 0.5 ml. of serum. Jendrassik and Grof[7] measured bilirubin content by following the extinction of blue bilirubin azo dye formed by the addition of alkali using 0.25 to 0.5 ml. of serum. With[8, 9] introduced a micromodification of that method using 0.1 ml. of serum in normal and 0.2 ml. of serum in slightly hemolyzed specimens. Davidson, Merritt, and Weech,[10] in a study on physiological jaundice, adapted the method of Ernst and Forster[11] and measured bilirubin levels using 0.1 ml. of serum. This last method is of limited clinical application because of the length of time required for acetone precipitation and the quantitative precipitation of the direct bilirubin by acetone which prevents the measurement of that fraction.

METHODS AND MATERIALS

The present method consists of collecting blood in capillary tubes and determining both the one-minute and total bilirubin levels on 0.05 to 0.1 ml. of serum by a modification of the method of Malloy and Evelyn using the microcell attachment of the Evelyn colorimeter.

Reagents.—(a) Solution A. 1.0 Gm. of sulfanilic acid dissolved in a liter of water containing 15 ml. of concentrated hydrochloric acid. (b) Solution B. 0.5 per cent sodium nitrite. (c) Diazo reagent is prepared fresh each day by adding 0.3 ml. of solution B to 10 ml. of solution A. (d) Diazo blank is prepared by adding 15 ml. of concentrated hydrochloric acid to 1 liter of water. (e) Absolute methyl alcohol.

Apparatus.—(a) Evelyn photoelectric colorimeter with microcell attachment.* (b) Two microabsorption cells, 2 ml. open type.* (c) Capillary tubes 8.0 cm. in length and 0.25 cm. in internal diameter which are chemically cleaned. These are fitted with rubber caps.† (d) 0.1 ml. pipettes calibrated in units of 0.01 ml. (e) 10 ml. centrifuge tubes.

Procedure.—1. *Collection of Blood:* (a) Blood is collected in the capillary tubes by heel puncture using the tip of a No. 11 Bard Parker blade. In most instances, one full capillary tube yields 0.1 ml. or more of serum. In normal newborn infants during the first twenty-four hours of life, one and one-half to two tubes are sometimes needed because of high hematocrit. Better flow of blood can be obtained by soaking the infant's heel in a pan of warm water or rubbing the infant's foot vigorously with an alcohol sponge prior to heel puncture. (b) The specimen is capped with the rubber caps and can be centrifuged immediately at 1,500 r.p.m. for fifteen minutes. The serum is separated from the cells by

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*Manufactured by Rubicon Co, Philadelphia.

†Manufactured by West Co. Phoenixville, Pa., Catalogue No. V3-68MS orange.

filing and breaking at the serum-cell boundary. With experience, no serum is lost in this process. The serum is then transferred to the 0.1 ml. pipette or recapped and stored in the icebox for future use.

2. *One-Minute and Total Serum Bilirubin Determinations:* (a) 0.1 ml. of serum is added to 0.9 ml. of distilled water in microcell A. If less than 0.1 ml. of serum is used, the volume is made up with distilled water to give a total volume of 1.0 c.c. in the cell. (b) 0.5 ml. of this solution is pipetted to microcell B. (c) 0.2 ml. of diazo blank solution is added to microcell A. This blank is placed in the micro attachment of the Evelyn photoelectric colorimeter and using a 540 μ filter the galvanometer reading is adjusted to 100. (d) 0.2 ml. of diazo reagent is added to microcell B and a reading is taken at the end of one minute.

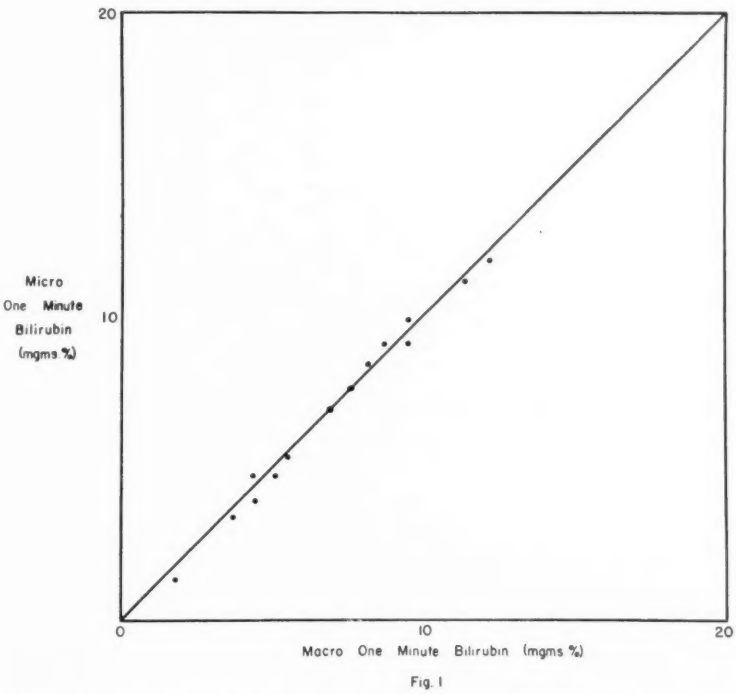

Fig. 1.—Results of duplicate determinations of one-minute bilirubin using the micromethod and the standard macromethod.

(e) The contents of microcells A and B are transferred to two separate centrifuge tubes. Two lots of 0.3 ml. of absolute methyl alcohol are used to rinse each of the microcells and transferred to the respective centrifuge tubes. (f) At the end of thirty minutes, 1.0 ml. of the contents of each of the centrifuge tubes is pipetted to the microcells, and the total serum bilirubin reading taken in the same manner. (g) The one-minute bilirubin and total bilirubins are each calculated by the use of the following formula:

$$X \text{ equals } \frac{2 - \log g}{3} \times 100$$

where X is the mg. of bilirubin per 100 ml. of serum, and
g is the galvanometer reading.

3. *Total Bilirubin Determination:* In some instances such as in erythroblastosis fetalis, only the total serum bilirubin determination is of importance. The following procedure can then be used with much saving of time. (a) 0.1 ml. of serum is added to 0.9 ml. of distilled

water in centrifuge tube A. (b) 0.5 ml. of this solution is pipetted to centrifuge tube B. (c) 0.2 ml. of diazo blank and 0.6 ml. of absolute methyl alcohol is added to centrifuge tube A. (d) 0.2 ml. of diazo reagent and 0.6 ml. of absolute methyl alcohol is added to centrifuge tube B. (e) 1.0 ml. of the contents of each tube is pipetted to its respective microcell at the end of thirty minutes and the readings taken. (f) The results are calculated in the same manner.

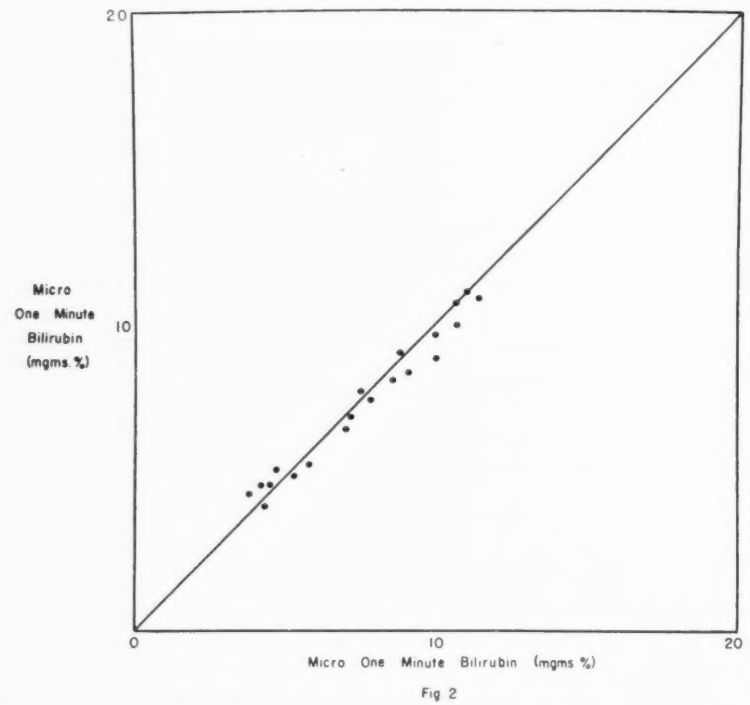

Fig. 2.—Results of duplicate determinations of one-minute bilirubin using the micromethod.

RESULTS

Fig. 1 shows the results obtained by the micromethod as compared with the standard macromethod of Malloy and Evelyn[5] for one minute serum bilirubin on duplicate specimens. The standard deviation of the difference was ± 0.38 mg. per cent, with a correlation coefficient of 0.9991.

Fig. 2 shows the results obtained by performing duplicate determinations using the microtechnique for one minute serum bilirubin. The standard deviation of the difference was ± 0.44 mg. per cent, with a correlation coefficient of 0.9983.

Fig. 3 shows the results obtained by the micromethod as compared with the standard macromethod for total serum bilirubin on duplicate specimens. The standard deviation of the difference was ± 0.61 mg., with correlation coefficient of 0.9993.

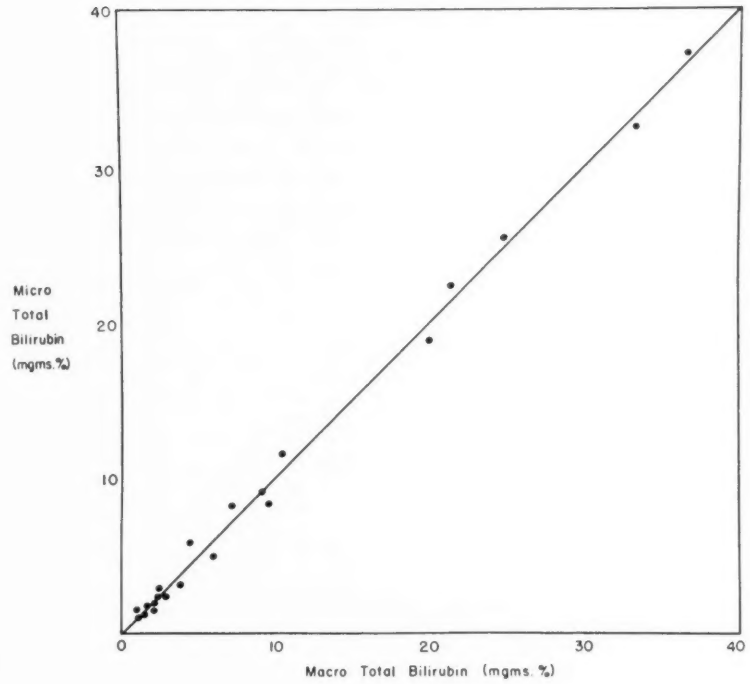

Fig. 3

Fig. 3.—Results of duplicate determinations of total bilirubin using the micromethod and the standard macromethod.

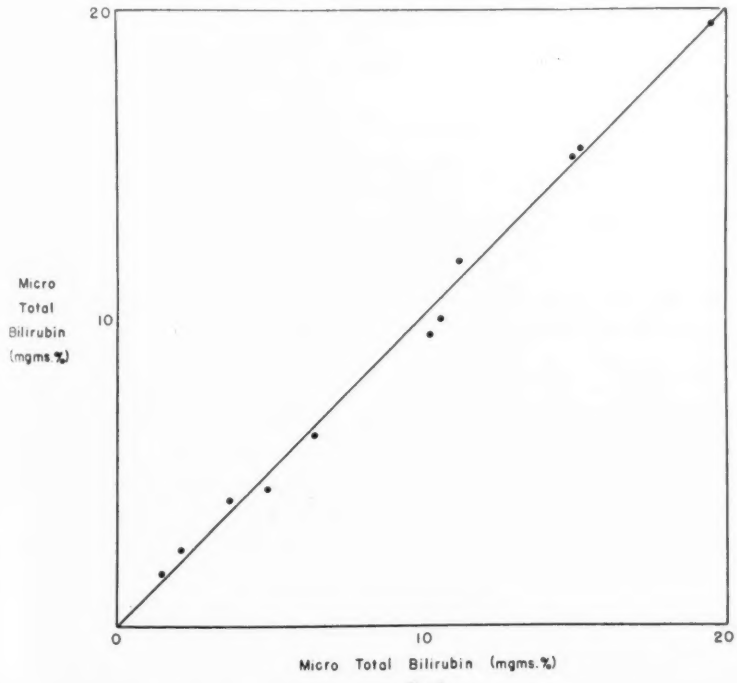

Fig. 4

Fig. 4.—Results of duplicate determinations of total bilirubin using the micromethod.

Fig. 4 shows the results obtained by performing duplicate determinations using the microtechnique for total serum bilirubin. The standard deviation of the difference was ± 0.32 mg. per cent, with a correlation coefficient of 0.9993.

Fig. 5 compares the venous and capillary samples obtained simultaneously in newborn infants using the micromethod for total serum bilirubin. Capillary blood averaged 0.07 mg. per cent less than venous blood. The standard deviation of the difference was ± 0.86 mg. per cent, with a correlation coefficient of 0.9909.

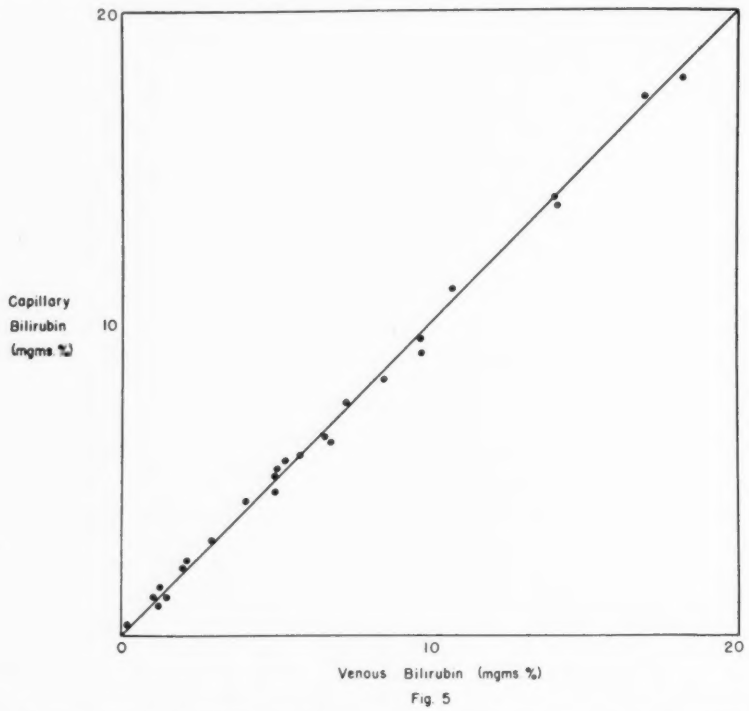

Fig. 5.—Results of simultaneous determinations of total bilirubin with venous and capillary blood using the micromethod.

The use of this technique in studies on physiological jaundice[12] and erythroblastosis fetalis[4] will be reported in subsequent publications.

SUMMARY

1. A micromodification of the method of Malloy and Evelyn using capillary blood for the determination of serum bilirubin levels has been presented.

2. Duplicate results of one-minute and total serum bilirubins by the micromethod check satisfactorily with the standard macromethod.

3. The correlation between capillary and venous bilirubin levels is presented.

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MEASUREMENT OF POLYVINYLPYRROLIDONE CONCENTRATIONS IN BODY FLUIDS BY MEANS OF A TURBIDITY-PRODUCING REACTION WITH PHENOL

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WE OBSERVED in connection with studies of the effect of polyvinylpyrrolidone (PVP) on liver function that serum containing this substance gave turbidity in excess of that expected when tested with the thymol reagent of MacLagan[1] or the phenol-sodium chloride reagent of Kunkel, Ahrens, and Eisenmenger.[2]

The turbidity produced by the latter was especially striking. Further tests showed that it was the phenol and not the sodium chloride component that was responsible. The turbidity seemed to depend upon the concentration of PVP in serum, being most noticeable shortly after infusion and gradually declining thereafter. This reaction thus appeared to offer a basis for a quantitative method for determining PVP in body fluids.

Several methods have been proposed for PVP determination in body fluids. The most widely used is that in which PVP is precipitated by trichloracetic acid after removal of protein by means of kaolin or ferrocyanide. The precipitate is extracted with methanol or water. The nitrogen content of the extract is determined by a micro-Kjeldahl method.[3, 4, 5, 6, 11] Other methods for determination of PVP include: a turbidimetric method dependent upon the turbidity produced by addition of sodium sulfate after removal of proteins by means of methanol,[7] a quantitative precipitation of PVP by iodine solutions,[8] the change in color of iodine-KI solutions induced by PVP,[9, 10, 15] and the change in the absorption spectrum of brilliant vital red caused by PVP.[12] All of these methods are time consuming with the possible exception of the sodium sulfate turbidity method.[7] However, the turbidity produced in this method is less than that caused by phenol with a given amount of PVP.

The phenol turbidity methods described in this paper are simple and rapid. The phenol solution may be added to urine or serum directly to produce the characteristic turbidity, or it may be added to serum filtrate. Each procedure has advantages, and for this reason two different techniques are described. The first is applied to urine or to serum without removal of protein. The effect of the serum proteins upon the turbidity produced by phenol is

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eliminated by the addition of the patient's serum to the standards. This method requires about two ml. of serum to prepare the standard, but is carried out on only 0.1 ml. of serum.

In the second technique, protein is removed and the standards may be prepared from any pooled serum. The determinations made by this method require 1 ml. of serum which suffices for duplicate estimations.

DETERMINATION OF PVP IN URINE

Phenol solution: Liquefy phenol, reagent quality, by addition of 10 ml. of water to 100 Gm. of phenol crystals. Dilute 1.5 ml. of the liquefied phenol to 100 ml. with water.

Phosphate buffer, pH 5.6: 93 ml. of potassium dihydrogen phosphate solution, M/15, and 7 ml. of disodium hydrogen phosphate solution, M/15. M/15 potassium dihydrogen phosphate: Dissolve 9.07 Gm. of KH_2PO_4 in water, and dilute to 1 liter. M/15 disodium hydrogen phosphate: dissolve 9.47 Gm. of Na_2HPO_4 in water. Dilute to 1 liter.

Stock Standard: Dissolve 500 mg. of PVP in 9.0 ml. of water. Add 0.5 ml. of merthiolate. Transfer to a 10 ml. volumetric flask and dilute to the mark with water.

Working Standards: Divide aliquots of the stock standards with urine obtained from the patient shortly before administration of PVP, or with pooled urine if the patient's urine is not available. Dilute 0.1 ml. of the stock (5 per cent) PVP standard with 0.9 ml. of urine to prepare a standard equivalent to 500 mg. of PVP per 100 ml. Prepare a standard of half this concentration by diluting 0.5 ml. of the 500 mg. per 100 ml. solution with 0.5 ml. of urine, and a third standard by diluting 0.5 ml. of the 250 mg. per 100 ml. solution further with 0.5 ml. of urine. The third standard represents 125 mg. per 100 ml. of urine.

Method: Measure 2 ml. of phosphate buffer, pH 5.6, into a cuvette or other suitable container. Add 0.5 ml. of urine (smaller volumes if the PVP concentration exceeds 500 mg. per 100 ml.) and 5.0 ml. of phenol solution. Mix and allow to stand fifteen to twenty minutes. Read in a photometer using a wave length of 660 mm. and water for the zero setting. The standards are treated the same way. Add 0.5 ml. of each standard to phosphate buffer of pH 5.6. Phenol reagent is added and readings are made as described. Standards are prepared and tested at the same time as unknown urine samples.

Once a standard curve has been established for a patient, two of the standings may be omitted.

Calculation: The absorbance of each standard is plotted against its concentration. The PVP concentration of the unknowns is calculated from the graph so obtained.

DETERMINATION OF PVP IN SERUM. A. WITHOUT REMOVAL OF PROTEIN

Phenol solution: As described under urine.

Acetate Buffer: pH 4.8. 37.5 ml. of acetic acid, 0.1N; and 62.5 ml. of sodium acetate 0.1M (8.20 Gm. per 1 liter).

Stock Standard: Dissolve 500 mg. of PVP in 9.0 ml. of pooled serum. Add 0.5 ml. of merthiolate. Transfer to a 10 ml. volumetric flask and dilute to the mark with pooled serum.

Standards: Prepare standards by diluting 0.1 ml. of the stock (5 per cent) PVP standard with 0.9 ml. of the patient's serum. This working standard is equivalent to 500 mg. per 100 ml. Prepare additional standards containing 250 and 125 mg. per 100 ml., by diluting with serum following the procedure described in the directions for urine.

Method: Measure into a cuvette or other suitable container, 2 ml. of acetate buffer. Add exactly 0.1 ml. of serum measured by means of a pipette calibrated "to contain." Rinse the pipette with the buffer solution. Add 5.0 ml. of phenol solution from a pipette. Mix the contents of the cuvette by shaking ten to fifteen seconds and read in a photometer as described under urine.

The standards are prepared and tested at the same time as unknown sera: 0.1 ml. of each standard is added to 2 ml. of the acetate buffer. 5.0 ml. of phenol reagent are added and readings are made as described.

Calculation: As described under directions for urine. Concentrations of PVP up to 1,500 mg. per 100 ml. may be measured by this method. If the concentration exceeds 100 mg. per 100 ml., 0.05 ml. of serum is used. It is necessary for accurate results to dilute the PVP used as standard with serum collected before PVP is administered.

DETERMINATION OF PVP IN SERUM. B. AFTER REMOVAL OF PROTEINS

Zinc sulfate solution: 100 Gm. of $ZnSO4 . 7H_2O$ dissolved in distilled water and diluted to 1 liter.

Sodium hydroxide solution: 0.5N.

Phenol solution: 1.8 ml. of liquefied phenol (see under urine) is diluted to 100 ml. with water.

Acetate Buffer, pH 4.0: 81.5 ml. of acetic acid 0.1N and 18.5 ml. of sodium acetate, 0.1M.

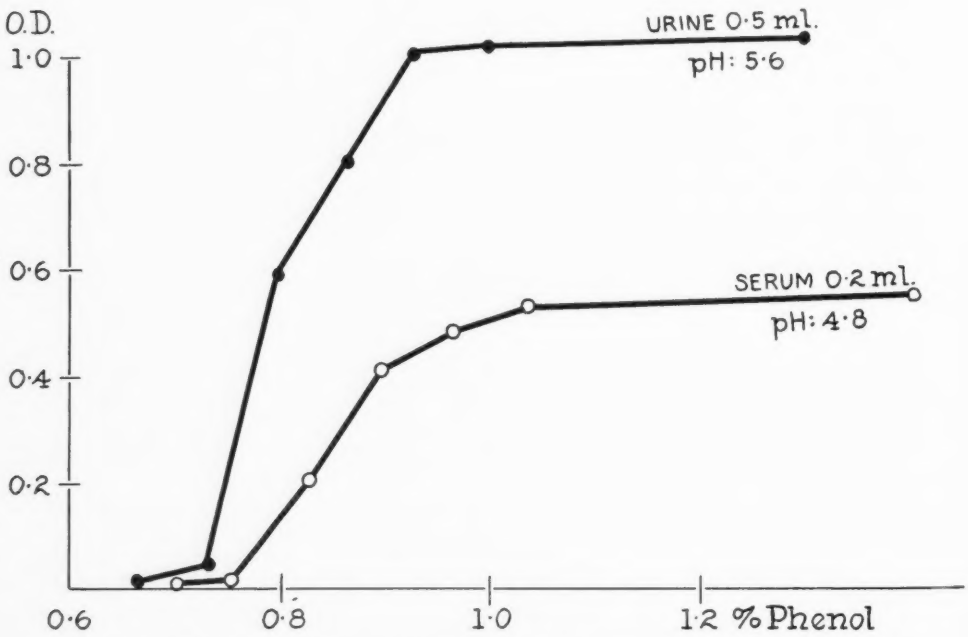

Fig. 1.—Effect of increasing concentration of phenol on turbidity produced with PVP. Phenol concentration is given in per cent of the final volume. The PVP concentration in urine and in serum was 583 mg. per 100 ml.

Stock Standard: Same as described for serum under method A.

Working Standards: Prepare these standards with pooled serum. Surplus serum remaining after completion of miscellaneous analyses may be used for this purpose. The pool should contain serum from not less than five individuals. *Standard I:* Dilute 0.5 ml. of the stock standard solution to 5.0 ml. with pooled serum; the concentration is 500 mg. per 100 ml. *Standard II:* Dilute 2.0 ml. of standard I with 2.0 ml. of pooled serum. This standard is equivalent to 250 mg. per 100 ml. *Standard III:* Dilute 2.0 ml. of standard II with 2.0 ml. of pooled serum. This represents 125 mg. per 100 ml.

Method: Measure 8 ml. of water into a flask of 50 ml. capacity. Add 1 ml. of serum and 0.5 ml. of zinc sulfate solution. Mix and allow to stand 2 minutes. Add 0.5 ml. of sodium hydroxide solution. Mix and again allow to stand. Filter.[13]

Measure 2 ml. of filtrate (1 ml. if the PVP concentration exceeds 500 mg. per 100 ml. and 1 ml. of water) into a cuvette or other container. Add 1 ml. of acetate buffer, pH 4.0, and 5 ml. of phenol solution. Mix and read in fifteen to twenty minutes in a photometer as previously described. Treat the standards in the same way as the unknowns.

Calculation: The same as described above.

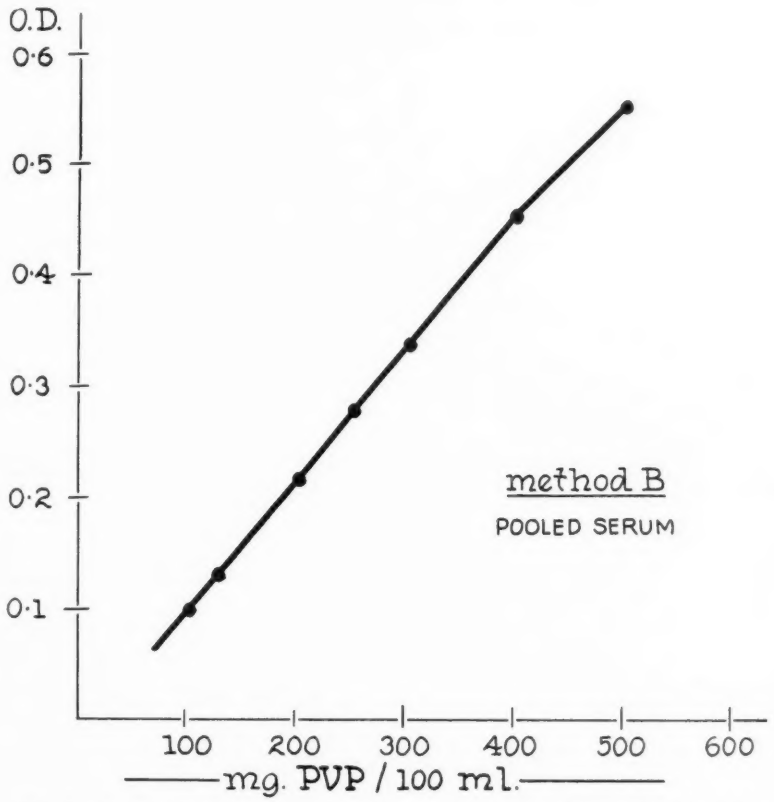

Fig. 2.—Turbidity produced by 5.0 ml. of 1.6 per cent phenol added to 2.0 ml. of serum filtrate and 1.0 ml. of acetate buffer of pH 4.0. The PVP concentrations in serum include those commonly encountered when PVP is used as a plasma expander.

EXPERIMENTAL

Optimal Concentration of Phenol.—Turbidity increased sharply with increasing concentration of phenol until the latter approximated 0.9 Gm. per 100 ml. of the reaction mixture. Additional phenol had only a slight effect (Fig. 1). The concentrations used in the several methods were selected accordingly. It is significant that the same phenol concentration gave maximal turbidities independently of the PVP concentration. The decreased PVP solubility manifested by turbidity production thus appears to be caused by a salting-out effect rather than a chemical reaction with the phenol.

Concentration of PVP.—Fig. 2 shows the relationship between PVP concentration in serum filtrates and absorbance for method B. Values between 100 and 400 mg. per 100 ml. fall on a straight line of satisfactory slope. Above

400 mg. per 100 ml. the slope decreases slightly. Three standard solutions, 125, 250, and 500 mg. per 100 ml. define the relationship sufficiently well for most purposes.

In the presence of serum, as exemplified by application of Method A, the absorbance is directly related to concentration up to 500 mg. per 100 ml. (Fig. 3). Curves for urine are shown in Fig. 4.

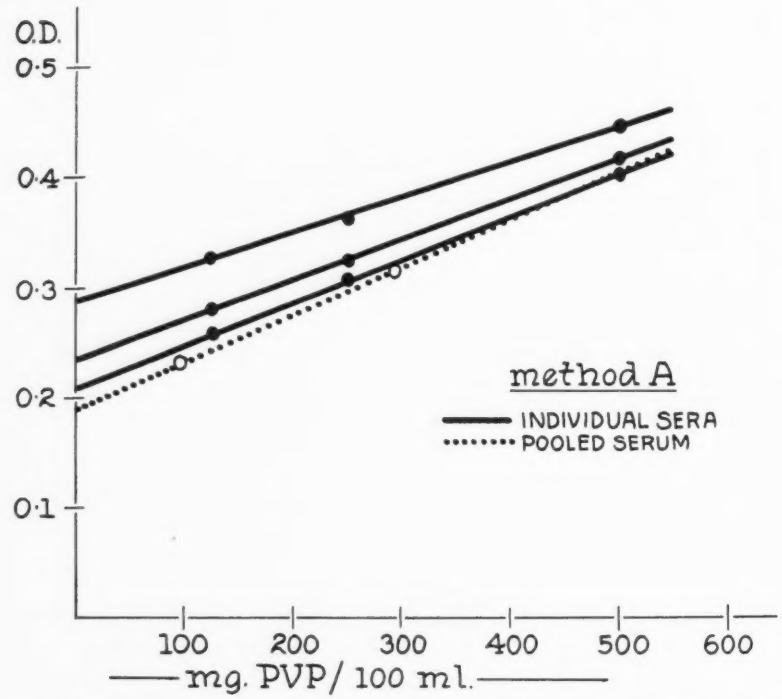

Fig. 3.—Turbidity produced when 5.0 ml. of 1.35 per cent phenol solution is added to 2.0 ml. of acetate buffer pH 4.8 and 0.1 ml. of serum containing different amounts of PVP.

Effect of pH.—pH had an important influence on the degree of turbidity and its reproducibility. The optimal pH differed in aqueous solutions, serum, and urine. In aqueous solutions the best reproducibility was obtained by addition of M/15 phosphate buffer of pH 7.8. In urine M/15 phosphate buffer of pH 5.6 was superior to acetate buffer at the same pH. For serum tested by Method A, acetate buffer of pH 4.8, and by method B, of pH 4.0, gave superior results. Serum alone also gives maximal turbidity with phenol at the same pH of 4.8 (method A). However, attempts to carry out the test at a pH giving low serum blank readings were unsuccessful due to poor reproducibility of the phenol PVP turbidity.

Effect of Molecular Size.—PVP having K-values of 23 and 24 produced less turbidity per unit of weight than did preparations of greater molecular size

(Table I). The PVP preparations tested were dissolved in water, urine, or serum. The results are similar in each of the three solvents. Urines from two individuals and two serum pools were tested. At K-values of 44 and higher, differences in turbidity were relatively small. It is desirable therefore, that the PVP used as a standard approximates the K-value and composition of the PVP administered. For urine analyses, PVP for use as standards should have preferably a K-value of 20 to 30 in order to approximate the

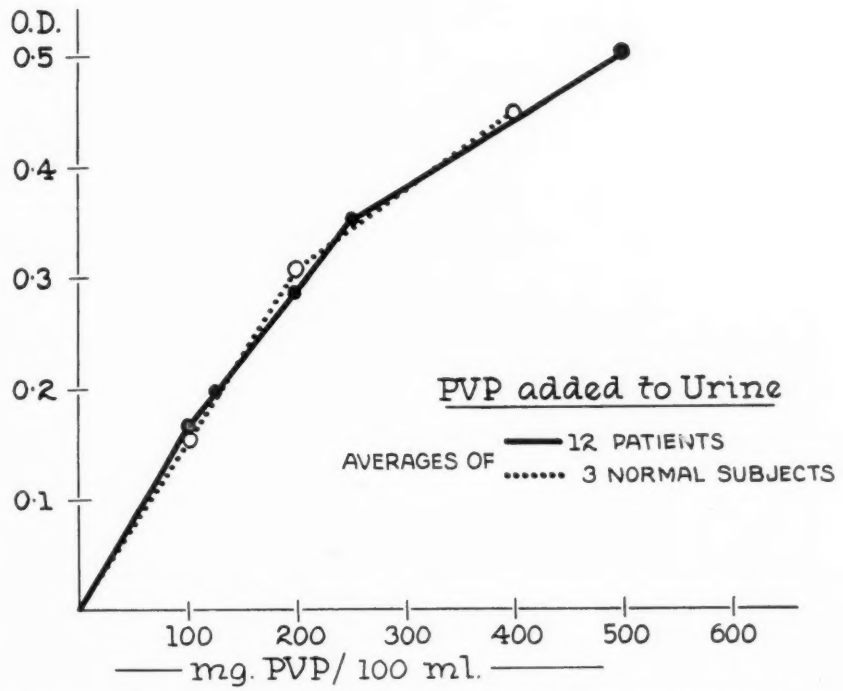

Fig. 4.—Turbidity produced by addition of 5.0 ml. of 1.35 per cent phenol to 2.0 ml. of phosphate buffer, pH 5.6, and 0.5 ml. of urine containing varying amounts of PVP. The behavior of patients' urine is similar to that of healthy subjects. One exception is described in the text.

smaller molecular size of excreted PVP. If this is not available and PVP of higher K-value is to be used in preparation of standards, the urine results may be adjusted with the aid of the relationships shown in Table I.

Influence of Drugs.—Urines of 14 patients representing a random sampling of a hospital population receiving a variety of medications were tested with the phenol solution after addition of PVP sufficient to give concentrations of 125, 250, 500, and 1,000 mg. per 100 ml. (Fig. 4). In 13, the curves obtained when absorbance was plotted against concentration showed close agreement with those observed when six urines of normal subjects were tested. The exception was a patient receiving terramycin. After standing in the refrigerator one week, this urine behaved as did the others.

TABLE I. THE INFLUENCE OF THE K-VALUE UPON THE TURBIDITY PRODUCED WITH PHENOL REAGENT

BODY FLUID		PVP ADDED IN MG./100 ML.	ABSORBANCE K-24	K-44	K-62	K-87
Urine	1	500	0.908	1.119	1.119	1.053
	2	500	0.783	1.059	1.072	1.023
	1	250	–	0.709	0.711	0.820
	2	250	0.557	0.733	0.719	0.745
	1	125	0.138	0.285	0.272	0.274
	2	125	0.223	0.288	0.320	0.385
Serum, Method A						
Pool	1	500	0.489	0.525	0.518	0.511
	2	500	0.444	0.463	0.475	0.482
	1	250	0.393	0.415	0.415	0.408
	2	250	0.349	0.345	0.363	0.384
	1	125	0.357	0.361	0.372	0.357
	2	125	0.285	0.300	0.320	0.317
Serum, Method B						
Pool	1	500	0.498	0.579	0.545	0.563
	2	500	0.497	0.575	0.586	0.573
	1	250	0.254	0.288	0.288	0.299
	2	250	0.265	0.301	0.298	0.299
	1	125	0.117	0.136	0.134	0.139
	2	125	0.127	0.154	0.145	0.150

Recovery of PVP Added to Serum.—The method for urine and Method A for serum enabled recovery of added PVP to be made within 10 per cent of the expected values at concentrations up to 1,500 mg. per 100 ml. (Table II).

TABLE II. RECOVERIES OF PVP

PVP ADDED IN MG./100 ML.	2,400	1,500	1,000	700	500	400	300	200	100
Recovered from:									
Urine	2380		910		550			220	110
Serum, Method A		1452	950	665	501	420	330	213	
		1590	990	703					
		1630	925	673					
Serum, Method B		1282	995	800	486	389	292	195	
		1255	995	788					
		1250	960	745					

Method B gave recoveries within 3 per cent of the theoretical for concentrations up to 500 mg. per 100 ml. It was not reliable at concentrations exceeding 500 mg. per 100 ml.

We wish to acknowledge the kindness of Dr. Bruno Puetzer of Schenley Laboratories, Inc., for providing PVP fractions of different K-values.

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THE ESTIMATION OF FREE GLUTAMIC ACID IN URINE

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OBSERVATIONS in this laboratory have indicated that the free glutamic acid content of blood plasma is increased in patients with malignancy and in certain other conditions.[1] To determine whether the increase results from diminished urinary excretion it is necessary to have available a reliable, specific method for the estimation of glutamic acid in urine. Microbiological procedures[2, 3] and an enzymatic method[4] have been reported. These methods do not differentiate between glutamic acid and glutamine. Because of low sensitivity, special conditions for growth of the organisms are required in the microbiological methods. A chromotographic technique has also been employed.[5]

The chemical procedure developed by Prescott and Waelsch[6] for use on blood plasma has been found to be reliable and specific, although it does not distinguish between the optical isomers. Considerable experience with this method led us to modify it for use with urine specimens.

METHODS

Preliminary Observations.—In the Prescott-Waelsch procedure[6] glutamic acid is converted to the acid aldehyde, β-formyl propionic acid. The 2,4-dinitrophenylhydrazone is produced from the aldehyde and is estimated colorimetrically.

In attempts to apply the Prescott-Waelsch method to urine directly it was found that coloring material, ordinarily present in urine, interfered with the estimation of the hydrazone. Several trials of decolorizing agents showed that acid-washed Norit A carbon was satisfactory and that no loss of glutamic acid occurred if the pH of the urine was adjusted to 4.7 to 5.2. It had been found previously that a normal absorption spectrum for glutamic acid was obtained with solutions at pH 5.2. Consequently, this acidity was selected as suitable for decolorization. If the pH of urine is adjusted to 5.2, removal of color with Norit is satisfactory, and the Prescott-Waelsch procedure can be applied to the decolorized urine.

Estimation of Glutamic Acid in Urine.—Initial sample volumes of 15 to 20 ml. of undiluted urine have been found suitable. The pH of the sample is adjusted to 5.2 with 5N sodium hydroxide solution and/or 1N hydrochloric acid. Change in volume of the specimen is recorded to obtain a correction factor. About 4 Gm. of Norit A acid-washed carbon is mixed thoroughly in the urine and the mixture filtered through Whatman No. 1 paper. As soon as 2 to 4 ml. of filtrate is available, it is transferred, after recording the volume, to a 15 ml. centrifuge tube and the pH is adjusted to 7.0 with 5N sodium hydroxide solution and 1N hydrochloric acid, using bromthymol blue as internal indicator. The volume is made up to 5.0 ml. by adding distilled water. To two aliquots, each of 2 ml., the Prescott-Waelsch plasma procedure[6] is applied as modified in a later report.[7] Optical density measurements of the final hydrazone solutions are made with a Coleman Junior Spectrophotometer set at a wave length of 420 μ. Zero setting of the instrument is obtained with a blank solution containing 5 ml. borate buffer solution, pH 10.0, plus 2 ml. 15N alcoholic potassium hydroxide. For a reagent blank 4 ml. of 0.5N acetic acid is carried through the entire procedure.

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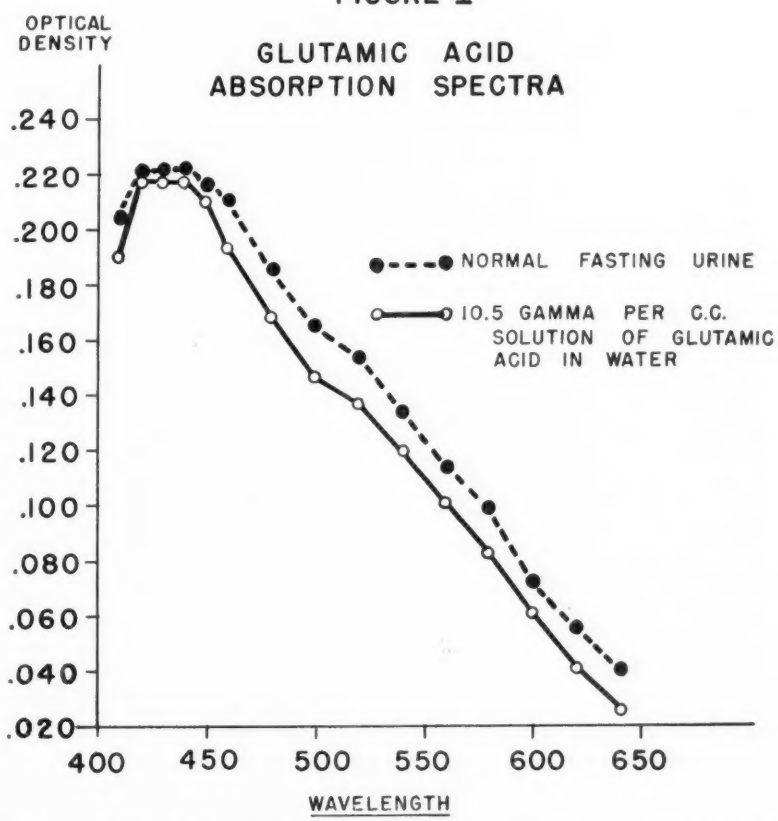
Optical density readings are converted to glutamic acid equivalents by the use of a standard curve prepared from the optical densities of solutions of the hydrazone of synthesized β-formyl propionic acid.[8]

For calculation of the glutamic acid content of urine the following equation is used:—

$$\frac{(A\text{-}B) \times 1.6 \times F.C. \times 5 \times C.F.}{2 \times \text{ml. filtrate}}$$

A = μg. glutamic acid read from the standard curve
B = blank reading given by acetic acid solution
1.6 = a factor derived from dilution of three aliquots at various stages (5/6 × 9/10 × 5/6)
F.C. = constant representing the retention of some glutamic acid on aluminum oxide and the distribution of the hydrazone between capryl aclohol and aqueous solutions; determined as described by Prescott and Waelsch.[8]
C.F. = correction factor due to volume change in initial pH adjustment.

FIGURE I



Precision and Specificity of the Procedure.—Recovery of known amounts of l-glutamic acid added to undiluted urine ranged from 98.5 to 99.5 per cent.

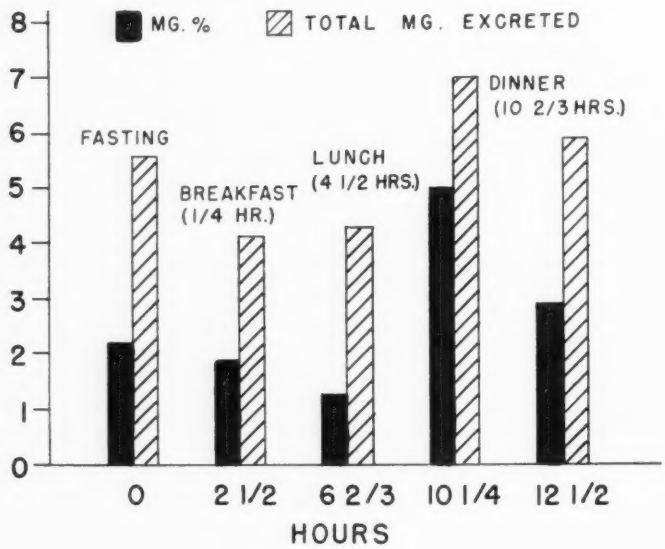
Absorption spectra of the hydrazone of glutamic acid naturally present in a urine specimen and of the hydrazone of l-glutamic acid in a prepared aqueous solution were obtained by plotting optical densities against wave lengths. The two curves are shown in Fig. 1. These curves indicate that the substance assumed to be glutamic acid in urine behaves similarly to l-glutamic acid. For both the urine constituent and pure l-glutamic acid the peak of intensity of the optical density of the hydrazone is between 420 and 440 μ.

Possible interference of several substances which may be present in urine was tested by adding individual compounds to solutions of glutamic acid of known strength and carrying these solutions through the complete procedure. Cystine, glutathione, glutamine, ammonium chloride, and aspartic acid were found to not interfere. Aspartic acid is completely separated from glutamic acid by the aluminum oxide adsorption step in the procedure.

The apparent glutamic acid content of urine specimens was found to increase during twenty-four hours storage and to subsequently decrease if urine was stored without a preservative in a refrigerator. If toluene is used as a preservative the glutamic acid concentration is constant for at least twenty-four hours' storage at low temperature.

FIGURE II

GLUTAMIC ACID EXCRETION
NORMAL SUBJECT—12 1/2 HR. PERIOD



RESULTS

Glutamic Acid Concentrations in Urine Specimens from Normal Subjects.—The described procedure has been used on 13 specimens obtained from 9 normal adults. In this limited series the range of values has been 1.1 to 3.1 mg. per cent, with a mean of 1.9. The results suggest that the concentration of glutamic acid in the urine of normal subjects in a fasting state is similar to that in the blood plasma and that there is no renal threshold for glutamic acid.

The excretion of glutamic acid by a normal female subject was followed through a period of 12.5 hours. The data obtained are shown in Fig. 2. It is evident that there is appreciable fluctuation in glutamic acid excretion and that two of three meals eaten during the period caused an increase in excretion.

These preliminary observations on human subjects are being extended in studies on patients with malignant growths before and after oral administration of glutamic acid. It is hoped to obtain information regarding the causes of increases in plasma glutamic acid above normal values.

It should be noted that the procedure has been used for the estimation of "free" glutamic acid in urine. Attempts to measure "bound" glutamic acid or glutamine have not been made.

SUMMARY

1. The procedure developed by Prescott and Waelsch for the estimation of free glutamic acid in blood plasma can be used on specimens of urine after decolorization with Norit at a pH of 5.2.

2. The procedure appears to be specific for glutamic acid and to be sufficiently precise for routine work.

3. Preliminary observations on 9 normal human subjects have been reported.

This study has been facilitated greatly by a grant from the National Cancer Institute of Canada, for which appreciation is expressed.

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BLOOD MEDIA FOR THE CULTIVATION OF MYCOBACTERIUM TUBERCULOSIS

IV. Effect of Adding Penicillin

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IN PREVIOUS studies[1-3] it was demonstrated that human bank blood incorporated into a variety of agar bases was capable of initiating early growth of small inocula of tubercle bacilli. The present study was undertaken to determine whether penicillin could be incorporated into these media for the purpose of preventing possible contamination which might result during the preparation of material for culture. It was also desired to ascertain whether the amounts of the antibiotic used, either alone, or in combination with the media, interfered in any way with growth.

MATERIALS AND METHODS

Two formulas of the blood media described previously [1-3] were used and are here designated as *A* and *B*.

A.	Agar-agar	1.5 Gm.
	Human bank blood	25 ml.
	Distilled water	74 ml.
	Penicillin (desired concentration)	1 ml.
B.	Blood agar base (Difco)	3 Gm.
	Human bank blood	25 ml.
	Glycerine (USP)	1 ml.
	Distilled water	73 ml.
	Penicillin (desired concentration)	1 ml.

Medium A was prepared by dissolving the agar-agar* in the water and sterilizing at 15 pounds pressure (121° C.) for fifteen minutes, after which it was allowed to cool at 45 to 50° C. in a water bath.

Two-day-old bank blood was then added which contained ACD solution† as follows: citric acid U.S.P. 0.50 Gm., sodium citrate U.S.P. 1.37 Gm., and dextrose U.S.P. 1.53 Gm. per each 100 ml. of transfusion solution. Finally sufficient aqueous crystalline penicillin G‡ was added in 1 ml. amounts to give final concentrations of 10, 50, and 100 units per milliliter. The flasks were kept in beakers of water in the bath to prevent tipping when the media became depleted during the tubing process. The flasks were shaken frequently to ensure adequate mixing and 3.5 ml. quantities of media were dispensed aseptically into sterile 15 by 125 mm. screw cap test tubes and allowed to harden into slants. Medium B was prepared by hydrating the blood agar base§ with the water containing the glycerine, and was completed in exactly the same way as described for Medium A. All media were tested for sterility prior to use. Both types of media were prepared without penicillin to serve as controls and for comparative purposes to be described in the experiments.

The H37Rv strain of *M. tuberculosis*‖ and 24-hour sputum specimens from tuberculous patients who had not received any therapeutic agents were used for study. The H37Rv organism was grown on Petragnani medium[4] prior to use, and inocula were prepared accord

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*Retort Pharmaceutical Co., Inc., Long Island City 1, N. Y., Lot No. 17Y.

†Baxter Transfuso Vac, Baxter Laboratories, Inc., Morton Grove, Ill.

‡Schenley Laboratories, Inc., New York 1, N. Y., Control 18S1338.

§Difco Laboratories Inc., Detroit, Mich., Control No. 393371.

‖Obtained from the Trudeau Laboratory, Trudeau, N. Y.

ing to the wet-weight quantitative planting method described by Corper and Cohn[5] using two drops of 0.5 per cent sodium taurocholate for initial grinding. Tenfold serial dilutions of the organism, using a single pipette for each dilution, were prepared in 50 ml. Erlenmeyer flasks containing the proper amounts of 0.85 per cent sodium chloride.

The sputa were concentrated by the trisodium phosphate technique,[6] and tenfold serial dilutions of the sediments were performed exactly as in the preparation of the H37Rv strain. The contents of each flask were thoroughly mixed prior to dilution and again at the time of inoculation of the medium. Each tube received an inoculum of 0.1 ml., and the order of inoculation of the media was varied at random. The tubes were incubated at 37° C. in a slanted position for the first 14 days to permit good distribution of the inocula and were then placed upright for the remainder of the period of incubation. All tubes were capped loosely for the first seventy-two hours to permit evaporation of excess moisture, and then tightened to prevent drying of the media. Readings were made daily under a bright light for six weeks. The day on which growth first appeared was recorded numerically, and the final degree of growth was indicated from + to ++++. Stained preparations of questionable colonies were examined microscopically. Each experiment was repeated once, and the final readings represent average results.

EXPERIMENTAL

The initial experiment was designed to determine the effect of penicillin on the growth of the H37Rv strain of *M. tuberculosis* on two blood media of different composition (formulas A and B). It was also desired to ascertain whether any significant differences in results would occur by the method of adding the antibiotic to the media. The experiment, therefore, was set up in duplicate. In Part I the varying concentrations of penicillin were added in 0.1 ml. amounts to each tube following inoculation and mixed thoroughly with the organisms. In Part II the penicillin was incorporated into the media. The data presented in Table I show that the rate and final degree of growth in all

TABLE I. GROWTH OF THE H37Rv STRAIN OF M. TUBERCULOSIS ON HUMAN BLOOD MEDIA. THE EFFECT OF ADDING VARYING CONCENTRATIONS OF PENICILLIN

MEDIUM	UNITS PENICILLIN PER ML.	MG. OF WET WEIGHT						
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
I. Control	0	4++++	4++++	4++++	4+++	6+++	10++	10++
	10	4++++	4++++	4++++	4++++	4+++	9+++	10++
Formula-A	50	4++++	4++++	4++++	5++++	5+++	10+++	10+
PII	100	4++++	4++++	4++++	4++++	4+++	8+++	10++
Control	0	4++++	4++++	4++++	4++++	9++++	10++	10++
	10	4++++	4++++	4++++	4++++	9++++	10+++	10++
Formula-B	50	4++++	4++++	4++++	5+++	5+++	10++	10+
PII	10	4++++	4++++	4++++	5+++	8++++	10++	10+
II.	10	4++++	4++++	4++++	4+++	5+++	10+++	10+
Formula-A	50	4++++	4++++	4++++	4++++	10+++	10+++	10++
PIM	100	4++++	4++++	4++++	4++++	5+++	10+++	11+
	10	4++++	4++++	4++++	4++++	5+++	10++	10+
Formula-B	50	4++++	4++++	4++++	4+++	10+++	10+++	10++
PIM	100	4++++	4++++	4++++	5+++	4+++	10+++	11+

Numbers represent the day on which growth was first observed.
Symbols + to ++++ represent final degree of growth after six weeks at 37° C.
Formula-A: Agar-agar plus 25 per cent human bank blood.
Formula-B: Blood agar base plus 1 per cent glycerine plus 25 per cent human bank blood.
PII: Penicillin in inoculum.
PIM: Penicillin in medium.

of the experimental tubes were essentially the same as in the controls, and no significant differences were observed due to the method of adding the penicillin to the media, or in changing the composition of the latter. The colonies on the medium made up according to formula B were larger, and somewhat easier to detect at times in the earlier periods of growth.

Since the results of the initial experiment were based upon the use of a standard laboratory strain of *M. tuberculosis*, it seemed desirable to determine if similar findings would be obtained with other virulent strains isolated directly from the sputum. For this purpose six such specimens were used and obtained from different patients. The blood medium was made up according to formula A, and the varying concentrations of penicillin were added in the same manner as described in Parts I and II of the previous experiment. The results presented in Table II are in agreement with those given in Table I.

TABLE II. GROWTH OF VIRULENT HUMAN TUBERCLE BACILLI ON HUMAN BLOOD MEDIUM-ORGANISMS ISOLATED BY SERIAL DILUTION OF CONCENTRATED SPUTUM SPECIMENS THE EFFECT OF ADDING VARYING CONCENTRATIONS OF PENICILLIN

SPECIMEN NO.		UNITS PENICILLIN PER ML.	SPUTUM DILUTIONS						
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
I. Control	PII	0	8++++	10++++	11+++	11++	12++	13+	15+
		10	7++++	10++++	10++++	10+++	11++	14+	17+
1		50	9++++	9++++	11+++	12++	11++	12+	15+
		100	8++++	10++++	10++++	11+++	11++	13+	16+
Control	PII	0	9++++	9++++	11+++	12++	12+	14+	17+
		10	8++++	11+++	11+++	12++	13++	13+	16+
2		50	8++++	12++++	12+++	13++	13+	14+	17+
		100	9++++	10++++	11+++	12++	13+	15+	17+
Control	PII	0	10++++	12++++	13++++	19+++	19++	0	0
		10	9++++	12++++	13++++	18++	20++	0	0
3		50	10++++	13++++	13++++	17+++	19++	27+	0
		100	10++++	13++++	14+++	17+++	19++	0	0
II. Control	PIM	0	11++++	12++++	12+++	17++	17++	21+	23+
		10	10++++	12++++	12+++	18+	18+	21+	24+
4		50	11++++	13+++	13+++	18+	19+	27+	23+
		100	12++++	12++++	13+++	19+	20+	21+	24+
Control	PIM	0	9++++	13+++	13+++	19+	0	0	0
		10	9++++	13++++	14++	19+	26+	0	0
5		50	10++++	13++++	13++	17+	0	0	0
		100	11++++	12++++	13++	19+	0	0	0
Control	PIM	0	9++++	12++++	13+++	13++	23+	37+	0
		10	10++++	13++++	13++++	14+	22+	39+	0
6		50	9++++	13++++	13++++	14++	21+	36+	0
		100	9++++	12++++	14+++	14+	19+	40+	0

Numbers represent the day on which growth was first observed.
Symbols + to ++++ represent the final degree of growth after six weeks at 37° C.
Medium used (Formula-A): Agar-agar plus 25 per cent human bank blood.
PII: Penicillin in inoculum.
PIM: Penicillin in medium.

Here again, the rate and final degree of growth in all the experimental tubes were essentially the same as in the controls. In two instances (specimens 3 and 5) growth occurred in a tenfold greater dilution on media containing penicillin than on the comparable controls, which is probably a result of experimental variation and not one of any stimulatory capacity of the penicillin. The periods at which growth was first detected were longer in this experiment than in the previous one, but this was attributed to the size of the inocula

which in the sputa sediments were considerably less than those prepared with the H37Rv strain. The relation of the quantity of the inoculum to rate of growth has been pointed out previously.[1, 5, 7] No contamination was observed either in the control or experimental tubes.

DISCUSSION

The reports concerning the effect of penicillin on the tubercle bacillus are contradictory. Abraham and associates[8] observed no inhibition of growth in a glycerol broth medium containing 40 units of penicillin per milliliter, and no antagonistic action by blood, serum, pus, peptones, or tissue autolysates on the antibiotic was noted. Smith and Emmart[9] showed that penicillin in concentrations of 100 to 3,000 units per 100 ml. of glycerine bouillon had no marked inhibitory effect on growth, and similarly that 50 to 1,000 units in Proskauer and Beck medium likewise exhibited no inhibition. Friedman[10] was able to obtain rapid growth of human, bovine, and avian strains of tubercle bacilli in embryonic tissue cultures containing 12 to 20 units of penicillin per milliliter, which had been added to inhibit contaminating organisms. Woodruff and Foster[11] noted that culture filtrates of tubercle bacilli were capable of destroying large amounts of penicillin (800 units per milliliter), which they attributed to the production of penicillinase, and the reason that *M. tuberculosis* was virtually insensitive to this antibiotic, they thought, was perhaps because it was one of the most active destroyers of penicillin. Illand[12] and Illand and Baines[13] found that recently isolated strains of *M. tuberculosis* grown in watery extracts of Lowenstein medium were inhibited by penicillin in strengths of 20 to 80 units per milliliter. Ungar and Muggleton[14] observed stimulation in growth of tubercle bacilli when small amounts of penicillin, up to 5 units per milliliter were added to the culture media. They felt the effect appeared to be due to the penicillin and not to the presence of impurities. Goldie[15] reported that penicillin concentrations of 0.5 to 2 units per milliliter in Dubos medium does not influence the growth of *M. tuberculosis*, and that these concentrations were capable of suppressing more or less completely contaminating organisms. Kirby and Dubos[16] demonstrated that small inocula of tubercle bacilli in Tween-albumin medium are highly susceptible to as little as 1 unit of penicillin per milliliter, but in oleic acid-albumin medium, a concentration as great as 100 units per milliliter causes no inhibition of growth. Similar results were reported by Smith and associates.[17] In their studies, Solotorovsky and associates[18] found that, depending upon the size of the inoculum, human, bovine and avian types of *M. tuberculosis* grown in Dubos medium were inhibited by penicillin varying from 1 to 200 units per milliliter. Investigating the diagnostic potential of a modified Dubos type medium without Tween 80, Zuckerman and Rantz[19] found that penicillin in a concentration of approximately 50 units per milliliter exerted no inhibitory effects on tubercle bacilli. Studying the value of penicillin in reducing contamination in sputum concentrates, Abbott[20] demonstrated that tubercle bacilli survive in this material treated with 100 to 200 units of penicillin per milliliter, and their recovery on Lowenstein-Jensen medium is not significantly affected.

The differences in the results of these reports, it appears, are due to the great variation in technique and to the selection of strains. The experimental data in this report confirm the observations of previous workers that under certain conditions penicillin in varying concentrations exerts no significant inhibitory effect on various types of mycobacteria, and this antibiotic has proved useful in cultivating tubercle bacilli from contaminated material.

The problem of contamination is often an important one in routine diagnostic work. While it might be assumed that the blood media herein described would be subject to greater contamination than egg media containing malachite green, the incidence of contamination on the former media was no greater than on the latter, despite the extensive manipulation required prior to inoculation.[3] It is felt that if proper precautions are exercised during concentration and inoculation, contamination is not likely to be a serious difficulty with the blood media. At times, specimens are received which are exceedingly difficult to rid completely of contaminating organisms, and, therefore, require special treatment. In those cases, such as laboratories receiving specimens through the mail which results in increased contamination, or where large-volume work is done, the use of penicillin should prove helpful. The value of penicillin as an adjunct in culturing tubercle bacilli from contaminated materials has been stressed previously.[16, 17, 19, 20]

No attempt has been made to determine how long the concentrations of penicillin used under the conditions of these experiments remain effective. However, Illand[12] demonstrated that in a water extract of Lowenstein medium, a concentration of 5 units per milliliter loses on an average 25 per cent in stregnth in 7 days at 37° C., and Abbott[20] showed that the action of penicillin in neutralized sputum concentrates endures from three to ten days in Lowenstein-Jensen medium at 37° C.

Although the rate and final degree of growth were essentially the same in both types of blood media studied, the colonies on the one made up according to formula B were larger, and somewhat easier to detect at times in the earlier periods of growth. Since the same observations were made on the control media, these differences cannot be attributed to any influence by the penicillin. Of interest also is the fact that in a previous report[3] formula A proved somewhat more efficient than formula B, and since the agar-agar used in this study was obtained from another manufacturer the results might, in part at least, be due to the composition of the agar. While previous experience with a variety of basal media gave comparable findings,[1] it is possible that different basal media, or different preparations of the same basal medium may produce variations in results. Additional experiments will be required, however, to elucidate the significance of this or other possible factors.

SUMMARY

1. In the experiments described it has been demonstrated that penicillin in concentrations of 10, 50, and 100 units per milliliter incorporated into human blood media, or added to the media after inoculation causes no inhibition of growth of tubercle bacilli even with the smallest inocula.

2. Although comparable results were obtained using two different formulas of the blood media, the colonies on the one made up according to formula B were larger and somewhat easier to detect at times in the earlier periods of growth. The possible explanations for these differences have been discussed.

3. The contradictory reports concerning the effect of penicillin on the tubercle bacillus, it appears, are due to the great variations in technique and to the selection of strains.

4. The value of penicillin as an adjunct in culturing tubercle bacilli, under certain conditions, from contaminated materials has also been pointed out.

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A NEW SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF CARBON MONOXIDE IN THE BLOOD

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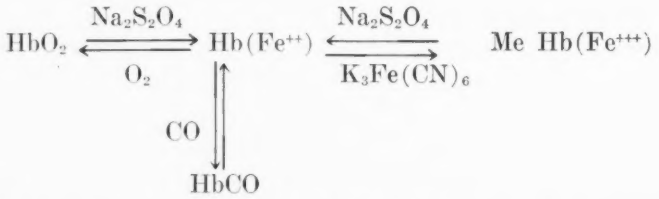
MANY techniques have been described for determining CO-hemoglobin in blood spectrophotometrically. Most of these involve absorption measurements at two or more wave lengths on hemolyzed blood diluted and treated with a reducing agent so that only reduced hemoglobin and CO-hemoglobin are present. It is possible from these measurements to calculate the proportion of pigment in combination with carbon monoxide. Measurements of this type are limited in their accuracy because of interference from extraneous pigments and variations in turbidity of the solutions, and because the measurements must necessarily be made, at least in the visible spectrum, at points where the slopes of the absorption curves of one or the other of the two pigments are quite steep.

A method has been developed which meets these objections and which requires no equipment that is not readily available in most clinical chemistry laboratories. It is much more rapid than the cumbersome gasometric techniques and is sufficiently accurate for the clinical determination of blood volume by the carbon monoxide method.

Kruse,[1] in 1921, first described the relative stability of CO-hemoglobin compared to oxyhemoglobin in the conversion to methemoglobin by ferricyanide, and suggested that this might be made the basis for an analytic technique. This idea has been developed and elaborated in the present method.

The reversible oxidation of oxyhemoglobin to methemoglobin proceeds via reduced hemoglobin[2] and CO-hemoglobin presumably follows the same pathway:



Reduced hemoglobin, when treated with ferricyanide, is converted to methemoglobin almost instantly. Oxyhemoglobin requires several seconds, and CO-hemoglobin takes several minutes for complete conversion to methemoglobin. If a mixture of oxyhemoglobin, reduced hemoglobin, and CO-hemoglobin, such

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as might be encountered in blood, is treated with sodium hyposulfite, the CO-hemoglobin is unaffected, and the oxyhemoglobin is converted to reduced hemoglobin. If then an excess of ferricyanide is added, the reduced hemoglobin is converted almost instantly to methemoglobin, and the CO-hemoglobin is converted slowly over several minutes. A photometric measurement taken immediately after the addition of the ferricyanide will give the optical density of the mixture of methemoglobin and CO-hemoglobin. A reading a few minutes later will give the optical density of the entire pigment as methemoglobin. This permits calculation of the amount of CO-hemoglobin present using the principle first proposed by Evelyn and Malloy[3] that if one component of a mixture of pigments in solution is altered the change in optical density of the solution is directly proportional to the concentration of the component which is altered. In this case the only change is the conversion of CO-hemoglobin to methemoglobin.

In actual practice the situation is more complex. For ordinary photometric determinations the blood must be diluted (1 to 200 in the present method), and in dilute solution the CO-hemoglobin dissociates to CO and hemoglobin. Since CO-hemoglobin is more stable in cold alkaline solutions, the fraction which dissociates is influenced by pH and temperature and these factors must be kept constant.

The fact that methemoglobin must be measured in the acid form limits the pH to the range between pH 6.1 and pH 7.5.[4] At pH 6.1 all the methemoglobin is in the acid form, but the CO-hemoglobin is quite unstable, decomposing rapidly and, more important, the fraction of CO-hemoglobin dissociated varies with concentration. At pH 7.2 90 per cent of the methemoglobin is in the acid form, the CO-hemoglobin is more stable, and fraction dissociated shows negligible change with change in CO-hemoglobin concentration. If at this pH the diluted blood sample is staturated with CO at atmospheric pressure, the dissociation is suppressed, and the amount of CO-hemoglobin in solution as determined photometrically is about 20 per cent greater than the value found in the absence of an excess of CO. This indicates that the fraction dissociated under these conditions is about 20 per cent. However, since this fraction is constant over the whole useful range of the determination, as indicated by the linear standardization curve (Fig. 2), it has no relevance in the determination except in regard to standardization.

A strong buffer (0.1M) was used because a large excess of ferricyanide was required to give a sharp initial reading. The strong buffer also provided a sufficient salt concentration to dissolve the globulins precipitated when the blood was diluted with distilled water, thus making the solution almost optically clear.

Examination of the absorption spectra of acid methemoglobin and CO-hemoglobin[4, 5] reveals two points where absorption measurements might be made, that is, where the curves are relatively flat and where there is a substantial difference in the absorption. One is at 565 mμ where there is a strong absorption peak for CO-hemoglobin and a flat plateau for methemoglobin. This proved unsatisfactory because the absorption of methemoglobin at this

point was very sensitive to temperature changes. The other point is at the acid methemoglobin peak at 632 mμ where CO-hemoglobin has a low flat absorption curve. The absorption at this point is perfectly stable to temperatures between 18° and 31° C., and both pigments obey the Beer-Lambert law up to concentrations of 1 Gm. per liter of hemoglobin on about 1/200 dilution of whole blood. At this or greater dilution the CO-hemoglobin determination is independent of the total pigment concentration.

Measurements were made with the Evelyn photoelectric colorimeter, which is an "abridged" spectrophotometer. When using this instrument, the "L values" calculated from the galvanometer readings are not strictly optical density measurements, but for practical purposes may be considered as such, and in the present paper will be referred to as such.

Since only differences in optical density are measured, it is unnecessary to set the instrument at 100 per cent transmission with a reagent blank. This permits reading the transmission differences on an expanded scale which increases the sensitivity of the instrument. The sensitivity can be further increased by mechanically depressing the zero point of the galvanometer a known distance. In this way the effective length of the galvanometer scale can be increased five or more times. This procedure is necessary when dealing with low CO-hemoglobin concentrations, and does not affect appreciably the galvanometer calibration. The fact that each sample acts in a sense as its own blank eliminates to a very large extent the usual random errors of photometric measurements and permits accurate measurement of much smaller differences in optical density than is usually considered feasible. Effects due to differences in turbidity, extraneous pigments, reagents, and imperfections in the cuvettes all cancel out, as the only factor operating to change the optical density is the change of CO-hemoglobin to methemoglobin.

METHOD

Reagents:

20 per cent w/v $K_3Fe(CN)_6$ in distilled water	10 c.c. made up fresh daily
$Na_2S_2O_4$	powdered reagent
1M phosphate buffer pH 7.2	102.3 Gm. Na_2HPO_4 (anhyd.) and 38.1 Gm. KH_2PO_4 (anhyd.) are dissolved in 1 liter of solution. pH when diluted 1 to 10 should be 7.20. This should be checked against a standard buffer using a pH meter and adjustment should be made for any deviation greater than 0.02.

Blood for determination of CO content is drawn from a vein without stasis and placed in a bottle containing 25 mg. of dry potassium oxalate per milliliter of blood. If not used immediately it should be stored in the cold under oil. For the determination, 0.5 ml. of blood is pipetted accurately into a 100 ml. volumetric flask containing about 85 ml. of distilled water and mixed by swirling. Exactly one minute is allowed for hemolysis, and then 10 ml. of buffer are pipetted rapidly into the flask and 25 ± 5 mg. of powdered hyposulfite are added with a calibrated spoon.[6] The solution is made up to volume and

TABLE I. CO DETERMINATIONS ON SAME SAMPLE IN PRESENCE OF VARIOUS INTERFERING CONDITIONS

VOLUME % CO	INTERFERING CONDITION
2.89	None
2.86	Incomplete hemolysis
2.86	Lipemic serum
2.82	Jaundiced serum Bilirubin 6.8 mg. per cent
2.90	Evans Blue 0.48 mg. per cent

mixed without excessive shaking. Precise timing and rapid manipulation are necessary in these steps, and exposure to bright light should be avoided. It is important to add the buffer before the hyposulfite, as the latter is unstable in the unbuffered solution and gives rise to a colloidal precipitate. The diluted buffered blood is stable for several hours if kept stoppered in volumetric flasks, but must be discarded immediately after withdrawing aliquots for determination.

The 635 mμ filter is used in the Evelyn photoelectric colorimeter. The galvanometer damping is adjusted so that the indicator comes to rest within 3 or 4 seconds (2 oscillations), and the zero point is depressed 200 scale divisions so that 100 on the scale is actually 300 on the extended scale. When taking readings the instrument is set with the indicator at 100 with no sample in the instrument (center setting) and allowed to warm up until the drift becomes slight. For blood containing less than 12.0 Gm. per cent of hemoglobin, a lower center setting is required. 10.0 ml. of the diluted buffered blood at 25 ± 1° C. are pipetted into a colorimeter tube and placed in the well of the instrument, being careful to avoid excessive exposure of the solution to air. The indicator of the galvanometer at this point goes off the top of the scale. 0.2 cc. of the ferricyanide solution, approximately a fiftyfold molar excess, is then pipetted rapidly into the tube, and the contents of the tube are mixed by bubbling air through the solution gently for one second. Changes in galvanometer readings due to residual air bubbles persist for six to eight seconds in the absence of a foaming agent such as Saponin cause little difficulty. The galvanometer reading falls immediately after the addition of the ferricyanide, and then becomes almost constant for ten to fifteen seconds, giving ample time to take the initial reading. The in-

TABLE II. REPRODUCIBILITY OF CO DETERMINATION ON SAME BLOOD SAMPLE

	VOL. PER CENT CO CONTENT	MEAN
Dilution No. 1	2.86	
	2.89	2.88
	2.88	
	2.87	
Dilution No. 2	2.82	
	2.87	2.83*
	2.82	
	2.81	
Dilution No. 3	2.92	
	2.86	2.89
	2.88	
	*3.06	
Dilution No. 4	2.90	
	2.93	2.89
	2.88	
	2.83	
Dilution No. 5	2.85	
	2.87	2.88
	2.90	
	2.89	
Mean	2.87	2.885
Standard deviation	±.03	±0.005

*Omitted from mean.

dicator then drifts slowly down as the CO-hemoglobin is converted to methemoglobin and the optical density increases. Between five and ten minutes after the addition of the ferricyanide, the conversion is complete and the final reading is taken after checking the instrument for drift. It is important to agitate the tube before taking the final reading so that any sediment which may have settled out will be evenly distributed in the solution and thus comparable to the initial reading.

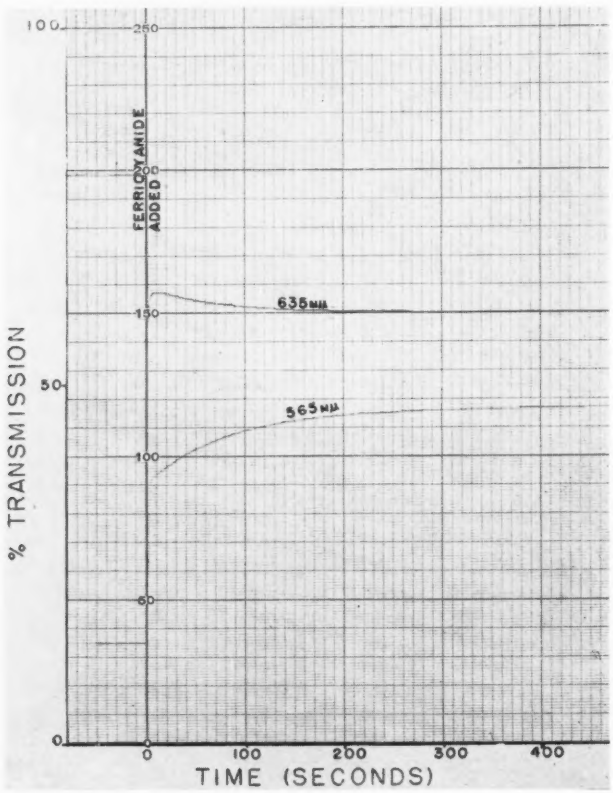

Fig. 1.—Changes in optical transmission of a hemoglobin–CO–hemoglobin solution following addition of ferricyanide to concentration of 0.4 per cent. (1/200 dilution of whole blood of O_2 capacity 17.7 volume per cent, CO content 2.08 volume per cent in 0.1M pH 7.20 phosphate buffer at 25° C.)

The calculation of the difference in optical density from the two galvanometer readings may be simplified. Since the optical density $L = \log G_0 \overline{G}$

$$= \log G_0 - \log G$$

where G = galvanometer reading of unknown and G_0 = Galvanometer reading of blank,

$$\triangle L = L_1 - L_2 = (\log G_0 - \log G_1) - (\log G_0 - \log G_2)$$
$$= \log G_2 - \log G_1$$

Remembering that the galvanometer readings lie between 200 and 300, the difference of the logarithms of the galvanometer readings gives the difference in optical density.

In Fig. 1 is shown the objective record of such a determination obtained with the Brown recording galvanometer. The blood sample had an O_2 capacity of 17.70 vol. per

cent and a CO content of 2.08 vol. per cent. The instrument was set at 250 (100 per cent transmission) with a reagent blank. Tracings were taken both at 635 mμ and 565 mμ, the former showing the appearance of new methemoglobin, and the latter showing the disappearance of CO-hemoglobin. It is of interest that CO-hemoglobin begins to disappear immediately after addition of the ferricyanide while there is a delay of ten to fifteen seconds before the appearance of new methemoglobin commences. This delay appears regularly, and is shorter at higher CO-hemoglobin concentrations. It may indicate the existence of some transitory intermediate substance. The delay serves a useful practical purpose in the determination, as it allows time for the initial photometric reading so that extrapolation to zero time is unnecessary.

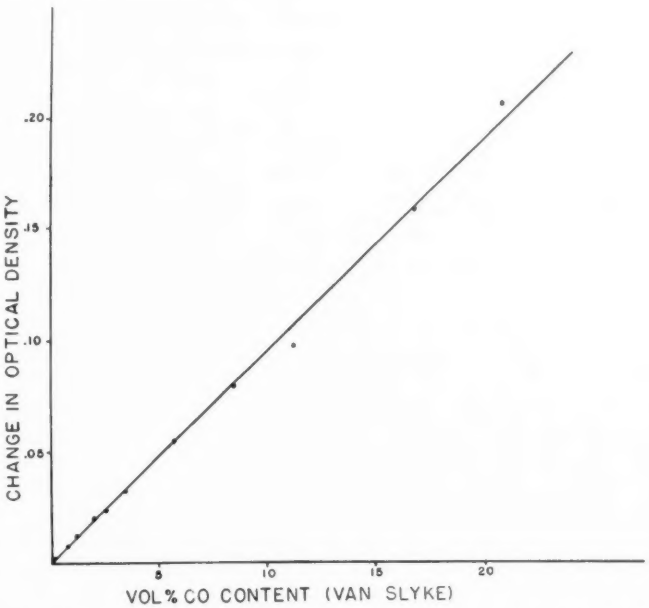

Fig. 2.—Standardization curve of spectrophotometric method against Van Slyke gasometric method (O_2 capacity of blood 20.7 volume per cent).

Standardization of the method for accurate work should be against the more precise gasometric technique[7] (see Fig. 2), which with our particular apparatus gave the following relation:

$$\text{Vol. per cent CO} = K\triangle L \text{ where } K = 108.2 \pm 1.3 \text{ at } 25^\circ \text{ C., pH } 7.20.$$

A rough standardization may be made photometrically using a blood sample of known hemoglobin content, expressed as Vol. per cent O_2 capacity. This is read against a reagent blank as methemoglobin, prepared as in the determination. The above factor may then be found from the relation $K = \frac{1.35 \times \text{Vol. \% } (O_2 \text{ cap.})}{L \text{ MeHb}}$. This involves the assumptions that relative absorption of methemoglobin and CO-hemoglobin are constant from apparatus to apparatus under the conditions of measurement, that the dissociation of the CO-hemoglobin is the same and that the cuvettes are the same size. In the absence of turbidity and extraneous pigments it should have no serious error.

The method may be applied to the entire range of saturation as shown in the standardization curve, although the precision is greater below 50 per cent saturation. Freedom from interference from extraneous pigments and varying degrees of turbidity is demonstrated in Table I.

Reliability as calculated from the data presented in the standardization curve gives a mean deviation of 0.05 vol. per cent from the gasometric determination for the eight points below 50 per cent saturation with CO. At higher saturations the absolute deviations became greater but the mean per cent deviation was only 3 per cent. These points all represent the average of triplicate photometric determinations, and duplicate gasometric determinations. The accuracy of a single photometric determination (standard deviation) is usually better than ± 0.1 vol. per cent, and with precise timing and manipulations a high degree of reproducibility may be obtained as shown in Table II. The ease and rapidity of the method are well illustrated by the fact that this entire series of measurements was completed in less than one hour.

Since only 0.05 ml. of blood is used in each determination, it is feasible to adapt the technique of finger-tip blood. The method should also be applicable with minor modification to use with the Beckman and Coleman spectrophotometers.

SUMMARY

A simple rapid photometric technique is presented for the determination of CO-hemoglobin in the blood using the Evelyn photoelectric colorimeter. No special apparatus is required and an accuracy (standard deviation) of 0.03 vol. per cent is attainable. There is no demonstrable interference from jaundice, hemolysis, lipemia, methemoglobinemia, or Evans Blue.

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PRACTICAL ASPECTS OF LABORATORY SCINTILLATION COUNTING

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THE physical characteristics of various phosphorus, photomultiplier tubes, and circuits suitable for scintillation counting have been covered in considerable detail in the literature in the last few years.[1-3] Reports have also appeared from this laboratory and others, on the clinical application of scintillation counters in the determination of I^{131} "uptake" by the thyroid gland, for obtaining in vivo scintigrams of radioactive areas, etc.[4-7] However, relatively little has been published concerning the practical details involved in performing radio-assays in the laboratory with a scintillation counter used as the radiation detector. It is the purpose of this report to present in some detail the scintillation counter techniques employed in this laboratory for the detection of beta particle and gamma ray radiation.

EQUIPMENT

A thallium-activated sodium iodide [NaI(Tl)] phosphor 4.3 cm. in diameter by 1.8 cm. thick is used as the scintillating crystal for the detection of gamma radiation and a stilbene crystal of similar dimensions for beta particle counting. These crystal phosphors are mounted in an aluminum holder fitted at one end with a screw-on Lucite cap fitted with a rubber gasket (Fig. 1, *a*). The outer surface of this Lucite cap is machined and polished to correspond to the photocathode end of an RCA 5819 multiplier phototube. The window end of the aluminum holder for the NaI(Tl) crystal is milled so that the aluminum window is approximately 0.1 millimeter in thickness. The window end of the stilbene crystal holder consists of one or two layers of black paper held in position by a spring clip. The aluminum crystal holder is threaded so that it may be screwed into a brass tube 25 cm. long by 6 cm. in diameter (Fig. 1, *b*). Fig. 1, *c* shows the 5819 photomultiplier tube and built-in preamplifier which likewise fits into the brass tube.*

The complete scintillation unit as described is operated in conjunction with a Tracerlab Autoscaler or "100" scaler (Fig. 2). The voltage for the photomultiplier tube and the preamplifier tubes is supplied by the scaling circuit.

OPTIMUM OPERATING VOLTAGE

The optimum operating voltage range for the scintillation counters described in this report must be determined for each instrument. This voltage or range, as will be seen, is largely determined by the amplification characteristics and the tube noise of the photomultiplier. If a comparison is made between the operating characteristics of a Geiger-Müller tube and a scintillation counter, some similarities and some differences are observed. In both

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*The complete unit for gamma counting (excluding crystal) can be obtained from the Radio Biological Apparatus Company, Berkeley 2, Calif. The holder for the stilbene crystal was fabricated in the radioisotope laboratory. The phosphors may be obtained from a number of sources such as Tracerlab, Harshaw Chemical and National Radiac Companies.

instances a threshold voltage exists below which no background or sample count is observed. As the voltage is increased, the background or the sample count increases to a plateau value where only small changes in the count rate are observed with increased voltage. This represents a region of optimum operating conditions for sample counting. Finally, in both instruments a voltage region is reached where the count rate increases very rapidly. In the case of the G. M. tube, this results from a continuous discharge of the tube,

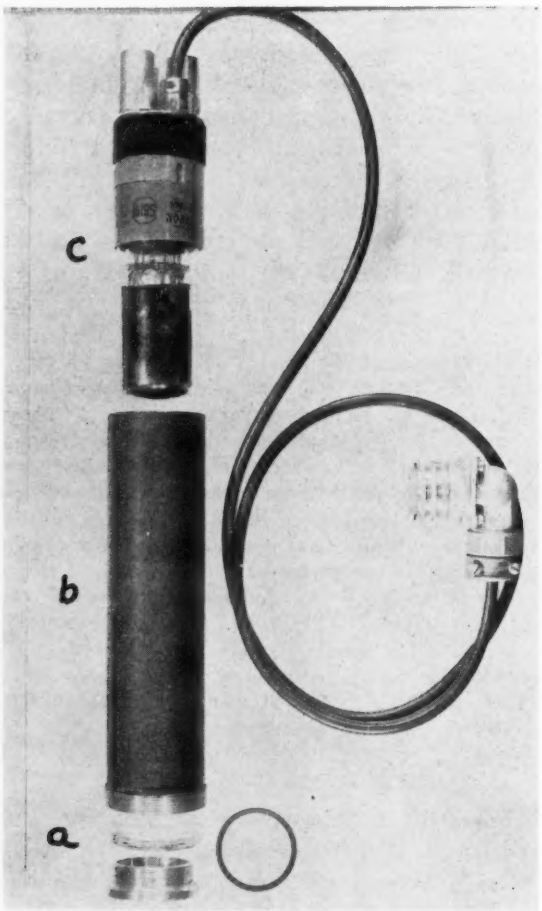

Fig. 1.—Components of Scintillation counter.

whereas for the scintillation counter this region represents tube noise or background due mostly to thermal electrons as distinct from crystal or G. M. background due to cosmic radiation or radioactive contaminants present in the lead chamber or other operational equipment.

Whether or not one obtains a true plateau region similar to that obtained with a G. M. tube in a gamma scintillation counter is dependent on several factors, the most important of which are: first, the voltage at which the tube

noise or background increases rapidly, and second, the energy of the gamma radiation. Fig. 3 shows a typical scintillation counter background curve in which counts per second are plotted against voltage. In this particular scintillation counter the crystal background threshold is 1,100 volts, the plateau region extends from 1,530 to 1,850 volts, and the tube noise background increases rapidly above 1,900 volts. The second short curve shown on Fig. 3 was obtained by interposing a piece of black photographic paper between the NaI (Tl) crystal and the photomultiplier tube. The tube noise background threshold in this instance was approximately 1,850 volts. Thus the observed background count between 1,000 and 1,850 volts represents a similar type of background count, i.e., cosmic radiation, etc., as that associated with G. M. counting. This will be further illustrated and amplified under the section of this report dealing with the effect of lead shielding on the crystal background count.

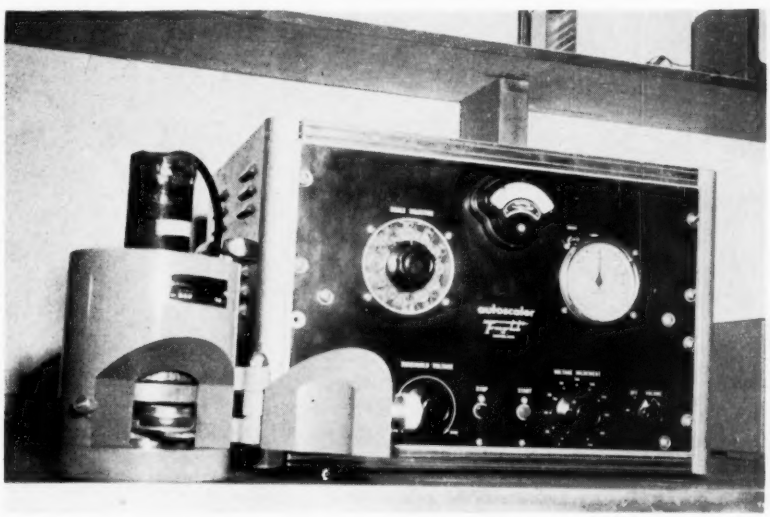

Fig. 2.—Operational setup for laboratory Scintillation counting.

The effect of the gamma ray energy of the isotope on the slope of the curve obtained by plotting the net counts per second vs. voltage is shown for Co^{60}, I^{131}, and Sb^{125} in Fig. 4. The gamma ray energies in MEV for Co^{60} are 1.17 and 1.33, for I^{131} 0.284, 0.364, and 0.638, and for Sb^{125} 0.431 and 0.609. As indicated in Fig. 4, the net counts for Co^{60} have a relatively steep slope between 1,450 volts and 1,520 volts; however, from 1,520 to 1,570 volts the count rate plateaus to a fairly constant count rate. By comparison I^{131} and Sb^{125} have steep slopes of about 23 per cent increase in count rate per 100 volts throughout the range of 1,450 to 1,570 volts. Since the optimum operating voltage for this scintillation counter is 1,550 volts, there is no plateau region of operation for these two isotopes. Beyond 1,570 volts the count rate increases very rapidly due to the contribution from tube noise background.

The fact that Co^{60} has a count plateau in the optimum operating range, whereas I^{131} and Sb^{125} do not, is directly related to the greater energy of the gamma radiation from Co^{60}. This in turn is related to the brightness or energy of the phosphor scintillation and the consequent increase in the voltage of the pulse from the photomultiplier tube. An additional indication of these energy relationships is the fact that Co^{60} has a lower threshold voltage than either I^{131} or Sb^{125}.

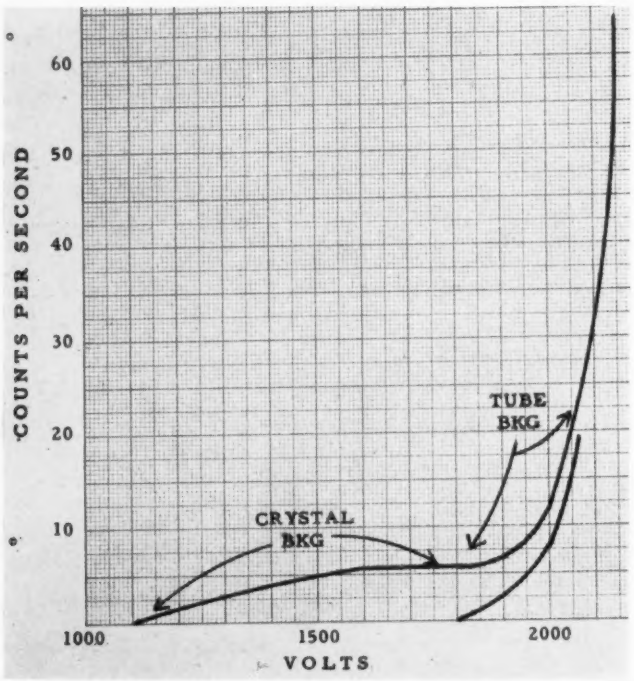

Fig. 3.—Crystal and photomultiplier background.

It should be made clear at this point that the voltages quoted and those shown in Figs. 3, 4, and 5 are read directly from the voltmeter on the scaling circuit. The true voltages on the photomultiplier tubes have not been measured. For all practical purposes this is not necessary.

The procedure used in this laboratory for determining the optimum voltage or range is illustrated in Fig. 5. The curves for Co^{60} and I^{131} are obtained by plotting the ratio of the net sample count to background vs. voltage. As indicated, the optimum operating voltage for this particular scintillation counter is approximately 1,120 volts. Here again, as in Fig. 4, the difference in response between CO^{60} and I^{131} with voltage change can be observed. The Co^{60} has an essentially flat ratio of net count to background plateau, whereas I^{131} has only a single voltage optimum at 1,120 volts.

It might be anticipated, on the basis of past experience using G. M. tubes as the radiation detector, that a single voltage optimum or the lack of a

plateau region would be a serious disadvantage in using a scintillation counter as a gamma radiation detector for I^{131} or Sb^{125}. However, in this laboratory over the past year quite the reverse has been true. For example, a Co^{60} reference source (38.7 counts per second) has been counted at least once every working day for the past six months. The standard deviation in the net count observed has been less than ±1 per cent during this period. This is the devia-

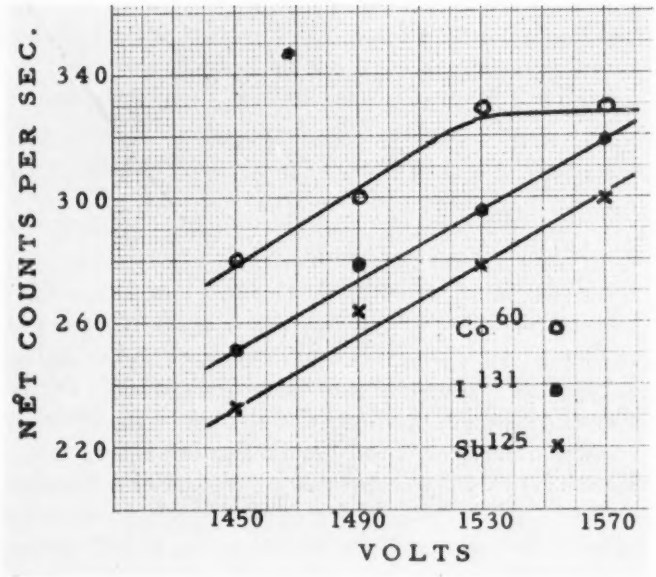

Fig. 4.—Effect of gamma-ray energy on the slope of net count vs. voltage curve.

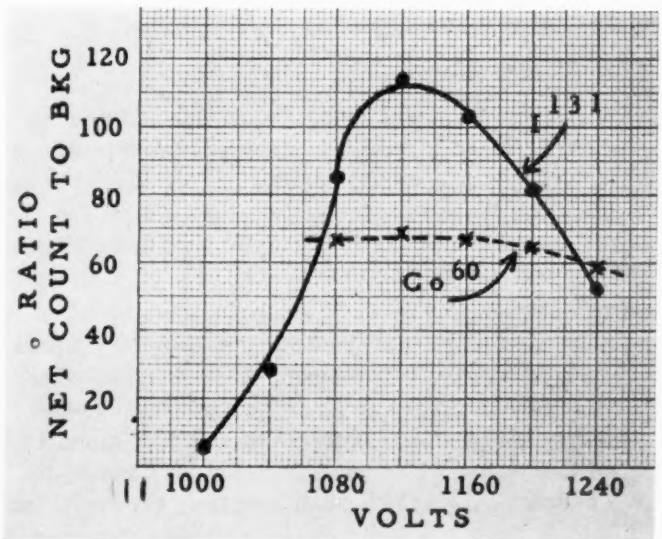

Fig. 5.—Determination of optimum operating voltage.

tion one would expect purely on the basis of the number of counts observed. The only operating procedure involved during this period has been to set the scaler operating voltage at 1,550 volts at the start of the work day.

No comparable records are available for a single sample of I^{131} because of its relatively short half-life; however, single samples of I^{131} counted over periods of three weeks gave standard deviations of the same order of magnitude. Additional evidence for the inherent stability of the scintillation counter is evidenced by the fact that weekly shipments of Oak Ridge I^{131} standardized by scintillation gamma counting have agreed within 1 or 2 per cent with the beta standardization of aliquots of the same shipment over the past eight months.

EFFECT OF SHIELDING ON CRYSTAL BACKGROUND

As pointed out in the previous section, the term crystal background is essentially synonymous with background radiation as associated with G. M. counting. Thus it is to be expected that shielding will lower the crystal background count. That this is true can be demonstrated as follows: with a 3.2 mm. lead collar around the NaI(Tl) crystal at a scaler voltage of 1,550, a crystal background of 15.2 counts per second is observed. When the counter with lead collar as described above is set flush with a ¾ inch thick lead disc, the count drops to about one-half, or 8.7 counts per second. If the scintillation counter with ⅛ inch lead collar is mounted in a lead chamber having ¾ inch lead walls and bottom, the crystal background drops to 3.96 counts per second (Fig. 2). If the sample door of the lead chamber is left open, the crystal background count increases to 5.3 counts per second.

Thus for increased accuracy in counting low activity gamma samples it is essential to mount the scintillation counter in a lead chamber in order to take advantage of the reduced crystal background.

SENSITIVITY TO GAMMA RADIATION FROM I^{131}

In general the same principles hold for scintillation gamma counting as those established for G. M. beta counting. Sample size, type of sample holder, distance to the detecting surface, and backscatter all affect the net count observed. Thus the data given in this section apply only for the operating conditions in this laboratory. However, they do have some significance as an indication of the sensitivity or efficiency of NaI(Tl) crystal counters for the gamma radiation from I^{131}.

A simple procedure has been evolved in this laboratory for liquid gamma counting. Five milliliter volumes of blood, plasma, urine or other solutions are pipetted into ointment tins (Fig. 2), 1⅞ inches in diameter by $1\frac{1}{16}$ inch deep, the tops are replaced and the tin set in a holder mounted in the lead chamber at a fixed distance of 16 mm. from the surface of the solution to the aluminum window of the scintillation counter. One tenth microcurie of I^{131} in 5 ml. of solution counted under the above geometrical conditions yields 217 net counts per second; this is a geometry of 5.9 per cent. This may be

compared with a geometry of 16.8 per cent for I^{131} beta counting (sample dried on a flat copper planchet) at the same distance from a Tracerlab 1.7 mg. per square centimeter mica end-window G. M. tube.

If the surface of the 5 ml. liquid sample in the ointment tin is moved to a 3 mm. distance from the aluminum window of the scintillation counter, the geometry can be increased to 11.7 per cent. Under these conditions as little as 0.001 microcurie of I^{131} in 5 ml. of solution can be counted in approximately ten minutes with a counting error no greater than ± 5.0 per cent.

RESOLVING TIME

Another advantage of scintillation counting derives from the fact that the range of counts per second can be extended far beyond those possible with G. M. counting without appreciable coincidence losses. The resolving time is limited only by the response of the scaling circuits and the maximum register speed and not by the radiation detector as in G. M. counting. Commercially available scaling circuits have resolving times of about 5 microseconds and will respond to 120,000 counts per minute with less than 1 per cent coincidence loss. No precise effort has been made to determine the coincidence losses in our scintillation counter. However, Table I shows the net counts per second and the counts per second per microcurie for a series of concentrations of I^{131} in 5 ml. of solution. As shown the net counts per second varied from 5,075 (304,500 counts per minute) to 651 counts per second. The mean value for the counts per second per microcurie was 2,170 with a standard deviation of ± 1 per cent. For the high counting rate samples an Eagle preset counter is used in conjunction with the Tracerlab Autoscaler so that counts of 40,960 to 81,920 are recorded. This minimizes any errors inherent in reading the seconds recorder.

TABLE I. RESOLVING TIME OF NaI(Tl) PHOSPHOR AND TRACERLAB AUTOSCALER

μc.I^{131}	NET COUNT/SEC.	COUNT/SEC. PER μC.	NET COUNT/MIN.
2.36	5075	2148	304,500
1.18	2591	2190	155,460
0.59	1279	2157	76,740
0.295	651	2186	39,060
	Mean	2170 ± 0.966 per cent	

REFERENCE STANDARD

Long-lived reference sources are required in gamma scintillation counting for normalizing counting rates just as they are in beta counting. The reference source when used as a counting rate standard will indicate when the counting apparatus is operating improperly.

A Co^{60} reference source was used for some time in this laboratory. However, as can be seen in Fig. 4, Co^{60} has some undesirable features as a reference standard. The main objection is that at the optimum operating voltage for scintillation counting Co^{60} has an essentially flat plateau, and thus it is relatively insensitive to voltage changes. Recently this laboratory has adopted Sb^{125} which

has a half-life of 2.7 years as a reference source for scintillation counting. The half-life of this isotope is reasonably long and, as indicated in Fig. 4, the slope of the count rate vs. voltage curve is identical with that obtained from I^{131} in the optimum operating range.

ADVANTAGES OF SCINTILLATION COUNTING IN THE LABORATORY

A scintillation counter has a much greater efficiency for penetrating radiation than the Geiger-Müller counter.[5] Thus, as might be expected, it has proved to be an exceedingly valuable instrument for the in vivo measurement of gamma emitting radioactive areas.[4-7] In laboratory sample counting it is possible to utilize this increased efficiency for the detection of gamma radiation to simplify counting techniques. Considerable time can be saved by counting liquid aliquots of blood, plasma, urine, etc., as outlined in the section on "Sensitivity to Gamma Radiation from I^{131}." In addition, the problems of self-absorption of the radiation within the sample are minimized or absent.

As indicated previously the day-to-day fluctuations in the count observed with a reference source have been less than those obtained with Geiger counting. In this laboratory the scintillation counter has had greater inherent stability than the G. M. tube.

Even though the initial cost of a scintillation counter is considerably greater than that of a G. M. detector, its life expectancy is unlimited. This is an important consideration in a radioisotope laboratory where all isotopes are standardized prior to their experimental or clinical use.

Another advantage of scintillation counting, as pointed out previously, is the ability of the instrument to respond to a much wider range of activities without coincidence losses (Table I), especially when it is well shielded.

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SIMULTANEOUS DETERMINATION OF PARA-AMINOSALICYLIC ACID AND PROMIZOLE

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AN INVESTIGATION was undertaken to find a method for the simultaneous determination of para-aminosalicylic acid (PAS) and Promizole when present concomitantly in serum or cerebrospinal fluid. Such a method is desirable due to the fact that Promizole is often used in conjunction with PAS in the treatment of tuberculosis. Since the amine group which is the functional group utilized for analysis is common to both drugs, it is to be expected that Promizole might interfere with the PAS methods which might be otherwise satisfactory, and vice versa. However, a Promizole method based on the Bratton and Marshall[1] procedure for sulfonamides was developed in which interference from PAS was so minimized as to be negligible. On the other hand, Promizole interference could not be eliminated in any of the PAS methods[2-4] tried. Two alternate approaches then were available for determining PAS under these circumstances, namely (a) removal of the Promizole by extraction, or (b) calculation of the optical density of the color due to Promizole in the most suitable PAS method and correction for this interference.

These approaches were tried, and satisfactory methods were worked out for both. Correcting for the interfering color is the simpler of the two procedures if the Promizole concentration is also desired, which is usually the case. On the other hand, the extraction method is preferred where only the PAS concentration is of interest.

METHODS

I. *Procedure for the Determination of Promizole or PAS in the Presence of Each Other.*—

A. *Determination of Promizole*[5]*:*

Reagents:

Trichloroacetic acid, 15 per cent.

Sodium nitrite, 0.1 per cent (prepared daily).

Ammonium sulfamate, 0.5 per cent.

N-(1-naphthyl)-ethylenediamine dihydrochloride, 0.1 per cent (kept in a dark bottle).

Stock Promizole standard, 10 mg. per cent. Dissolve 100 mg. of Promizole (crystalline) in 100 ml. of distilled water to which has been added 1.0 ml. HCl. Heat slightly to aid solution. Cool and make up to 1 liter.

Working standard, 4 mg. per cent. Dilute 40 ml. stock standard to 100 ml. with distilled water.

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Procedure:

1. To 7.5 ml. distilled water in a centrifuge tube, add 0.5 ml. of sample and mix. Prepare a standard and a blank in the same manner. To each tube add 2.0 ml. 15 per cent trichloroacetic acid slowly with mixing, allow to stand for 10 minutes and centrifuge.
2. Pipette 5.0 ml. of supernatant into an Evelyn tube and place in a 37° C. water bath for a minimum of 10 minutes.
3. Add 0.5 ml. of sodium nitrite, mix well, and allow to stand for 3 minutes.
4. Add 0.5 ml. of ammonium sulfamate, mix well and allow to stand for 2 minutes. The tubes may now be removed from the water bath.
5. Add 0.5 ml. of 0.1 per cent N-(1-naphthyl)-ethylenediamine dihydrochloride, and mix well.
6. Read immediately in the Evelyn colorimeter using filter No. 540 with the blank set at 100 per cent transmission.

Calculation: $\frac{\text{Optical density of sample}}{\text{Optical density of standard}} \times 4 =$ mg. per cent of Promizole in sample

B. *Determination of PAS:*

Reagents:

Trichloroacetic acid, 12 per cent.
Ethyl acetate.
Sodium hydroxide, 4 per cent.
Color reagent. To a mixture containing 90 ml. of 2 per cent Ehrlich's reagent in glacial acetic acid and 90 ml. of 4M sodium acetate in distilled water, add 40 ml. of 12 per cent trichloroacetic acid and 80 ml. of distilled water.
Stock PAS standard, 100 mg. per cent. Suspend 100 mg. of the pure acid in a little water, warming gently with 4 drops of 20 per cent sodium carbonate solution and adjusting the volume to 100 ml.
Working standard, 4 mg. per cent. Dilute 4.0 ml. of stock standard to 100 ml. with distilled water.

Procedure:

1. To 7.0 ml. of distilled water in a glass-stoppered 15 ml. centrifuge tube add 1.0 ml. sample and mix well. Similarly prepare a standard and a blank.
2. To each tube add 0.02 ml. of 4 per cent sodium hydroxide, adding a drop of phenolphthalein to make sure the solution is alkaline.
3. To each tube add 3 ml. of ethyl acetate and shake vigorously for two minutes. Centrifuge immediately and remove the ethyl acetate by aspiration. Repeat this step twice, adding 0.02 ml. of 4 per cent sodium hydroxide before each extraction.
4. To 4.0 ml. of the aqueous solution in a centrifuge tube add 2.0 ml. of distilled water and mix well.
5. Add 3.0 ml. of trichloroacetic acid, mix, allow to stand for 10 minutes and centrifuge.
6. To 5.0 ml. of the supernatant in an Evelyn tube, add 1.0 ml. of color reagent and mix.
7. After a minimum of 10 minutes, read the tubes in the Evelyn colorimeter using filter No. 470 with the blank set at 100 per cent transmission.

Calculation: $\frac{\text{Optical density of sample}}{\text{Optical density of standard}} \times 4 =$ mg. per cent PAS in sample

II. *Method for the Determination of Both Promizole and PAS in Mixtures (by Difference).*—

Reagents: Same as above.

Procedure:

1. Determine the mg. per cent of Promizole in the sample using the above method.

2. To 5.5 ml. of distilled water in a centrifuge tube, add 0.5 ml. of sample and mix well. Similarly prepare a PAS standard, a Promizole standard, and a blank.
3. Follow the above procedure for PAS starting with Step 5.

Calculation:

$$\frac{\text{Optical density of Promizole standard} \times \text{mg. per cent of Promizole in sample}}{4} = \text{O.D. from Promizole}$$

$$\frac{\text{Total O.D. of sample} - \text{O.D. from Promizole}}{\text{O.D. of PAS standard}} \times 4 = \text{mg. per cent of PAS in sample}$$

Promizole Determination.—The procedure for Promizole now in use at this laboratory[5] was investigated to see how much interference might be caused by PAS. This method consists of the diazotization of a trichloroacetic acid filtrate with nitrous acid, destruction of the excess nitrous acid with ammonium sulfamate, and coupling with N-(1-naphthyl)-ethylenediamine to give a stable red color which is compared with a standard treated the same way.

Under certain conditions PAS was found to give considerable interference in the Promizole method (e.g., PAS, weight for weight, gave about two-thirds as much color as Promizole at 5° C.). A number of variables in the method were investigated, and it was found that the interference from the PAS could be reduced by reducing the amount of trichloroacetic acid added to precipitate the protein, reducing the time of standing before reading the final color, and raising the temperature during color development. When the tubes containing the trichloroacetic acid filtrate were placed in a water bath at 37° C. for about 10 minutes before addition of the sodium nitrite and allowed to stay in the water bath until after addition of the ammonium sulfamate, the color produced by 20 mg. per cent of PAS in a number of determinations was equivalent to 0.06 to 0.16 mg. per cent of Promizole.

A series of Promizole standards was run at 37° C., and it was found that the curve followed Beer's law in the range tested (0.5 to 4 mg. per cent) and that the optical density value was no different from that obtained when the samples were run at room temperature. After color development, the color produced by the PAS intensifies on standing, while the color produced by the Promizole remains fairly stable, thus immediate reading of the tubes is necessary.

Since the PAS interference was so small when the samples were kept warm during color development, it was not felt to be necessary to change the amount of trichloroacetic acid added.

PAS Determination.—A number of methods have been proposed for the determination of PAS. These have been outlined in a review of PAS by Allen.[6] The procedures involving the free phenolic hydroxyl group, decarboxylation, and titrating the acidity were eliminated since they also determine the acetylated PAS which is believed to be biologically inactive.

The methods utilizing the free amine group appeared to be the most promising, and three of these were investigated: the methods of Way and associates[2] and Marshall[3] involving diazotization and coupling, and the method of Short[4] involving direct coupling with p-dimethylaminobenzaldehyde.

Short's method seemed to be simpler and more rapid than the other two methods tried, as well as having less interference from Promizole (see *Promizole Interference,* below), the only disadvantage being that at the concentrations expected the optical densities obtained with the available colorimeters were so low that the results were not reliable. A final dilution* of 1:20 instead of the original 1:50 was found to give accurate results. This is the lowest dilution that may be used, as Short states that at lower dilutions some of the PAS may be bound to protein, giving low results.

Promizole Interference.—Promizole interferes considerably in all three of the methods tried. Using Marshall's, Way's, and Short's methods, the optical density value of Promizole was respectively 75, 75, and 40 per cent that of the same amount of PAS.

Standard Curves.—In the range tested a linear relationship exists between optical density and the concentration for both PAS and Promizole. The Beckman spectrophotometer and

*The ratio of the concentration of PAS in the original sample to the concentration of PAS in the final solution.

the Evelyn colorimeter were used for this study, a wave length of 454 mμ being used on the Beckman and filter No. 470 on the Evelyn. It was decided to use the Evelyn with filter No. 470 as it is more practical for routine work and available in more laboratories.

Determination of PAS in the Presence of Promizole.—The total color produced by the sample may be measured, and with the simultaneous determination of a Promizole standard, a correction may be made for the color produced by the Promizole. This, of course, entails the separate determination of Promizole in the sample, with the described procedure.

The PAS content of a number of different mixtures of Promizole and PAS in pure solution was determined and the recoveries of PAS were between 93 and 106 per cent (average of 35 determinations on 6 different mixtures was 99 per cent).

Removal of Promizole.—In the event that the PAS level alone should be of interest, a separate procedure is presented for the determination of PAS after removal of the Promizole by ethyl acetate extraction.

About 90 per cent of the Promizole can be removed by three extractions with ethyl acetate. This should be sufficient for clinical purposes since the Promizole levels are usually much lower than the PAS levels, and Promizole gives only about 40 per cent of the optical density value of PAS.

In order to prevent the simultaneous extraction of PAS, it is necessary to make the solution basic before each extraction. However, it is important not to add too much base, as the pH at the time of final color development is very critical and an excess of base can reduce the color greatly.

To standardize such factors as pH of the solution and possible change in volume of the solution during extraction, the standard and blank must be treated similarly to the sample. Several mixtures of PAS and Promizole were run by this method, both pure solutions and solutions to which pooled human sera had been added. Recoveries of PAS ranged from 95 to 106 per cent (average 100 per cent).

Dilution of Final Sample.—If the sample contains a large amount of PAS, so that it does not fall within the range on the colorimeter which gives accurate results, it is possible to dilute it with a mixture equivalent to the blank. In a series of determinations with standard solutions containing PAS up to 20 mg. per cent, dilution of the final solution was found to give results exactly equivalent to those which were obtained with an equivalent amount of original solution, so that this technique may be used when the color is too intense. However, if a high result is expected, it would be advisable to use a smaller size of sample.

Protein Binding.—To ascertain that protein binding was not interfering with the results at the 1:20 final dilution, a series of PAS and Promizole standards, as well as of mixtures of the two, was prepared in serum and incubated for twenty hours at 37° C. and compared with solutions prepared in distilled water. To accomplish this, measured pure aqueous solution was put into Kahn tubes, and the water evaporated by means of a stream of air. Then an equal volume of pooled human sera was added to each tube, the tops sealed with aluminum foil, and the tubes incubated twenty hours at 37° C.

Promizole dissolved in serum (2 to 8 mg. per cent) gave recoveries of 100 to 103 per cent (average 101 per cent). PAS standards (2 to 20 mg. per cent) prepared in serum and incubated gave recoveries of 92 to 106 per cent (average of 27 samples run at four different times was 98 per cent). A series of ten 4 mg. per cent PAS standards run at different times gave recoveries of 96 to 106 per cent (average 99 per cent).

Recovery Study.—Mixtures of the two drugs were incubated with serum and the following recoveries of PAS were obtained:

mg. per cent PAS	2	2	4	4
mg. per cent Promizole	2	4	2	4
No. Determinations	8	8	12	6
Range (per cent)	92-97	81-94	84-93	86-95
Average (per cent)	95	88	90	92

In order to see at what dilution protein binding might be an appreciable factor, this was repeated using amounts of serum which would give a final dilution of 1:15, 1:12.5 and 1:10. At the 1:15 and 1:12.5 dilutions, the samples containing serum gave 94 to 101 per cent as much color as identical solutions with no serum. At the 1:10 dilution, however, the samples containing serum gave only about 80 per cent as much color, so it appears that protein binding may be appreciable at this dilution.

SUMMARY

Methods are described for the determination in serum or spinal fluid of para-aminosalicylic acid (PAS) and Promizole, either separately or in mixtures of the two.

Under the specified conditions PAS interference in the Promizole method can be rendered negligible. The Promizole interference in the PAS method has been eliminated through removal of Promizole by extraction or corrected by determination of Promizole and its equivalent optical density value as PAS.

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AN AUTOMATIC PIPETTE FOR PREPARING SERIAL DILUTIONS

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A MAJORITY of the time consumed in biological quantitation by the titration principle involves the preparation of serial dilutions of materials to be tested. The exacting and fatiguing nature of this operation ordinarily accomplished by pipetting with the aid of the mouth and index finger is well known to the biologist. To mitigate the tedium of this process, various mechanical contrivances have been devised. One of the first, perhaps, was the well-known Cornwall Pipetting Unit (No. 1251, Becton, Dickinson and Co., Rutherford, N. J.) which consisted of a spring-action syringe with a plunger activated by alternate pressure and release of a button. While effective in accomplishing mixing of solutions, the device had the disadvantage of drawing the fluid into the syringe itself; this procedure required that the syringe be washed between the different samples. A similar device without this undesirable feature was described by Svedmyr,[1] who added detachable glass pipettes, which were specially blown, to a metal syringe. A further improvement in the general principle was recently observed by one of the authors in the laboratory of Dr. Frank L. Horsfall, Jr. An ordinary 1 c.c. pipette was joined by means of a rubber hose to the Luer tip of the unmodified Cornwall Unit described above. The pipette was held at a right angle to the syringe in one hand, and effective and rapid dilution was accomplished with this arrangement. Despite the improvement, several minor objections remained, viz., some effort was required to insert the hose onto the pipette, and relatively large changes in the amount of fluid in the pipette resulted from minor changes in the relative position of the hose, syringe and pipette, thereby necessitating frequent adjustment of the delivery volume. The present communication describes additional improvements in the basic Cornwall Unit.

MATERIALS AND METHODS

Construction Detail.—The present automatic pipette consists of (1) a modified Cornwall Unit, (2) a copper air line with fittings, and (3) a bracket with pipette support. A photograph of the unit is shown in Fig. 1. Fig. 2 presents construction details; drawing I gives a cross section of the bracket and pipette support and a lateral view of the entire instrument is shown in drawing II. The regular Cornwall unit consists of a metal body tube (*L*) with finger supports (*T*) which accommodates a 2 c.c. syringe (*K*) with spring-activated piston (*M*). The plunger and assembly (*N-S* inclusive) of the regular Cornwall unit is modified as follows: the outer end of the syringe piston is ground to a smooth regular surface on an emory wheel. Plunger rod (*S*) with button (*R*) is shortened from 3½ inches to 2⅞ inches, and the volume-adjusting wheel (*Q*), which has a straight coarse knurl, is increased in size from 7/16 inch in diameter to 1½ inches in diameter. Cap (*N*) is rethreaded to ¼ inch–40 thread and a new screw (*O*) with the volume adjusting wheel (*Q*) is made. A friction spring (*P*) is inserted between the wheel (*Q*) and cap (*N*). The body tube (*L*) is inverted in position so that the finger supports (*T*) are forward.

The air line consists of a ⅛ inch copper tube (*E*) which forms an airtight passage between male-female connectors (*I-J*) and rubber hose (*D*). The air line is flanged on the proximal end and is equipped with a ⅛ inch brass female coupling (*I*) which engages a male

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connector (*J*). Male connector (*J*) fits the Luer-Lok (*U*) of the syringe (*K*) and is fashioned from a 14 gauge straight cannula 4 inches in length (A. S. Aloe Co., St. Louis, Mo., No. 69282) from which the tube is removed. The shank is threaded to accommodate female coupling (*I*) and bevelled to fit the flanged end of the copper tubing. The copper

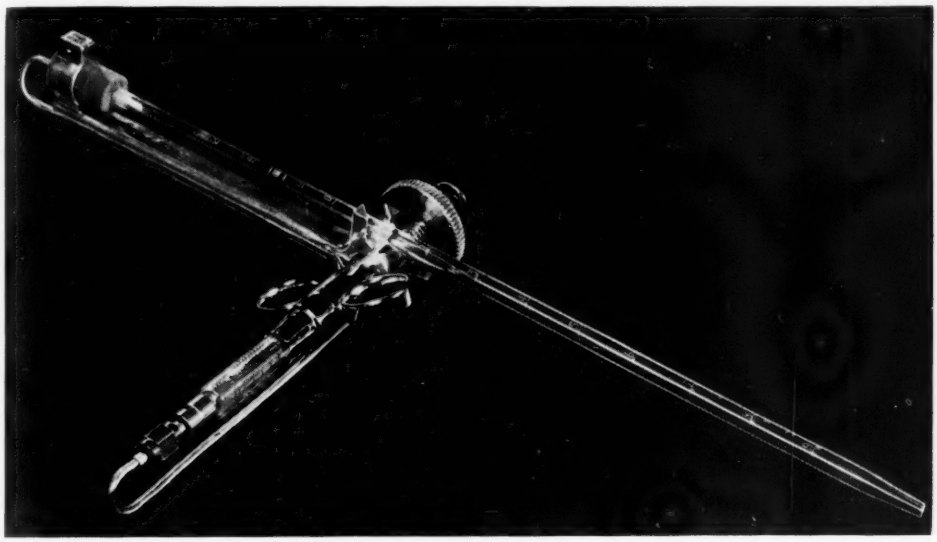
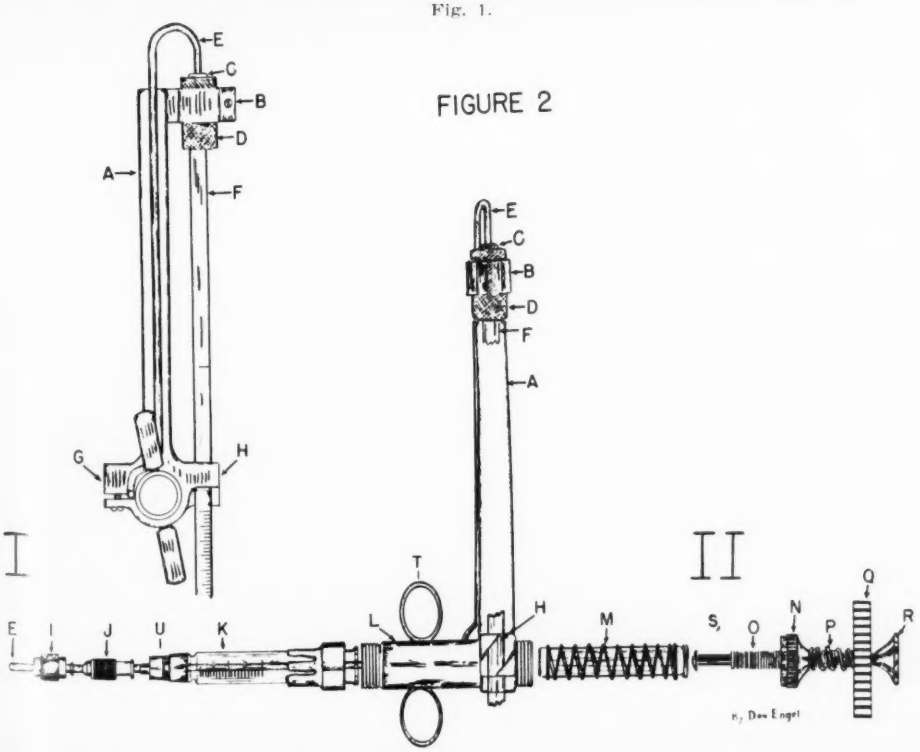


Fig. 1.

FIGURE 2



air line (*E*) is bent to parallel the syringe (*K*) and then follows the recess in one side of the bracket (*A*). The air line terminates in a soldered brass bushing 5/16 inch in diameter and ½ inch long, and inserts into a 1⅛ inch length of red rubber pressure tubing (*D*) with ¼ inch bore and 3/16 inch wall thickness. The rubber tubing (*D*) is held by clamp (*B*) which in turn is fastened to the bracket (*A*). The bracket is 6½ inches in over-all length and terminates on its lower end in a clamp (*G*) of ⅝ inch inside diameter which engages the body tube (*L*). The pipette support (*H*) is a twist-lock consisting of a vertical hole in the metal 0.295 inch in diameter and ½ inch long; this is milled through the outside at a 45 degree angle to permit introduction of graduated standard 1 c.c. pipettes (*F*). The hole in the pipette support coincides with the bore of the rubber hose so that the pipette is held parallel to the bracket. Wheel and adjustment screw (*Q* and *O*), and bracket (*A*) with clamp (*B*) and pipette support (*H*) are made of Duralumin. The syringe is lubricated with oil of light weight to prevent air leak.

The maximal transfer volume can be increased by enlarging (*H*) to accommodate a 2 c.c. pipette and lengthening the plunger rod (*S*).

Operation.—A calibrated 1 c.c. pipette is introduced into the pipette support (*H*) at a 45-degree angle and then rotated and inserted into tube (*D*). The second and third fingers are inserted into the supports (*T*) and the index finger is kept on the adjusting wheel (*Q*). The pipette is inserted into the first tube of the material to be diluted and the air is expelled by several quick thrusts on the plunger button (*R*) with the thumb. The desired transfer volume is obtained by rotating the wheel (*Q*). Mixing is accomplished by five or six steady thrusts on the plunger, and the serial transfers may be carried out either with the tubes left in the rack or removed individually. The pipette should be held at essentially the same angle to the bench throughout the operation in order to avoid the small changes in fluid volume which result from altered position.

DISCUSSION

There are several important advantages to be gained by use of an automatic device, such as described here, over the mouth and finger method. One of the more important factors has to do with accuracy. Error resulting from incomplete pipette drainage is prevented by stabilizing the height to which the fluid being tested is drawn into the pipette during mixing in the successive tubes. There is also less danger to the operator when highly infectious or toxic agents are being tested. Thus, the possibility of sucking the material into the mouth is eliminated, bubbling or foaming is minimized, and the serial dilution procedure can be carried out readily inside a closed hood with armholes.

The automatic instrument has been used in this laboratory chiefly for preparing serum or antigen dilutions for agglutination and agglutination-inhibition tests with influenza virus and for other serological work in which it is unnecessary to change pipettes for each successive dilution. The operation time required for preparing serial dilutions was decreased by about 50 per cent over the usual method, and there was considerable reduction in fatigue and eyestrain when large numbers of materials were processed.

SUMMARY

An improved automatic pipetting unit for preparing serial dilutions for titration purposes has been described.

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